THE ALKALINE PHOSPHATASE OF MILK

by

Walter Haab

ABSTRACT

The purpose of the present investigation was to study the variability and properties of alkaline phosphatase of raw milk. The method of Sanders and Sager was modified to provide a more accurate assay of enzyme activity.

Phosphatase activity of milk from four individual cows ranged from 70 to 4400 γ phenol during approximately one lactation period, whereas pooled milks varied between 950 and 1700. The possible relation between the enzyme and stage of lactation, physiological disturbances, milk yield, seasonal feed changes and fat content of the milk are discussed.

At 37.5°C. the optimum hydrolysis of disodium phenyl phosphate was defined by 0.029190 M substrate at pH 10.32 for a hydrolysis period of 20 minutes. The order of reaction, Michaelis constants and energy of activation were compared with the available data. Acid and alkali inactivation of phosphatase and partial restoration of activity lost by acid inactivation were confirmed. Magnesium ion in the concentrations added did not activate the enzyme. The inhibitory effect of sodium oxalate, sodium taurocholate and sodium cyanide on milk phosphatase varied with the specific compound and its concentration.

Factors involved in enzyme estimation and the nature of phosphatase which may possibly be important in the interpretation and evaluation of phosphatase tests for pasteurization are discussed.

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INTRODUCTION

During the past two decades evidence has been presented of the existence of alkaline phosphatase in cow's milk. This enzyme is called after its ability to hydrolyze certain phosphoric esters at an alkaline pH of the catalyzed reaction. Since the introduction of a phosphatase test for pasteurization by Kay and Graham (40) in 1935, the importance of the enzyme in the control of pasteurization has been universally recognized.

There is comparatively little information in the literature on the phosphatase activity of raw milk and possible variations in such activity. However, several tests based on residual phosphatase activity have been developed to detect irregularities in the pasteurization of milk and dairy products. The inconsistency of results from studies on time-temperature relation of phosphatase inactivation and bacterial death does not yet allow final conclusions to be drawn regarding the suitability of the phosphatase test for pasteurization control. This situation may be partly caused by lack of uniformity in experimental conditions and in the

reporting of results. An accurate procedure for the determination of phosphatase activity must provide for standardization of all factors which affect the activity of the enzyme, except the phosphatase concentration. Although the kinetics of the destruction of phosphatase by heat have received considerable attention, little information has been reported regarding the effects of such factors as: different substrates, substrate concentrations, pH, times and temperatures of hydrolysis, activators and inhibitors.

In this investigation the alkaline phosphatase activities of milks from individual cows and of pooled milks, as determined by a modification of the method of Sanders and Sager (59), were found to vary widely. Kinetic data for the enzyme were obtained. Certain of these results are fundamental to the accurate estimation of phosphatase activity and to the evaluation of reported data obtained by various modifications of the phosphatase test.

HISTORICAL

Occurrence and Nature of Phosphatase

In 1907, Suzuki et al. (71) found that certain cereals contained an enzyme which hydrolyzed phytin with the production of inorganic phosphate. Several similar enzymes have since been found to occur in various living tissues of both plants and animals. During the past four decades numerous investigations have established the presence of phosphatases in various secretions and excretions of the animal body including milk. In the review of Kay (38) are to be found references to the early literature on mammalian phosphatases. Folley and Kay (21) suggested a tentative scheme for classification of phosphatases according to occurrence or source of enzyme and the substrate hydrolyzed.

The milk of the cow normally contains phosphatases which catalyze the hydrolysis of certain phosphate esters in either alkaline or acid conditions. Wilson and Hart (74) were probably the first investigators to determine the alkaline phosphatase activity of bovine milk. Huggins and Talalay (32), Mullen (54) and Hakansson and Sjostrom (26) have reported on the occurrence and properties of acid phosphatase.

The possible presence of bacterial phosphatases in milk and dairy products has been studied by Tittsler et al. (72), Hammer and Olson (27), Tramer (73) and Sjostrom (67).

Little information is available on the alkaline phosphatase activity of individual raw milks and on factors causing variations in the activity. Wilson and Hart usually observed a higher phosphatase activity of milk than of the plasma of the cow. They reported the activity in milk to be much greater at the end of the lactation than at the beginning. Folley and Kay (20) found that there was a sharp drop from an initial high activity to a minimum between 10 and 25 days after parturition. Then phosphatase production increased to a peak at about 180 days after parturition followed by a gradual decrease to the end of lactation. Sanders and Sager, and Hetrick and Tracy (31) presented only limited data on phosphatase activity of pooled raw milks.

The origin of milk phosphatase has not been definitely established. Wilson and Hart determined the phosphatase activity of the milk as well as of the blood plasma of the cow. They suggested that there might be some elimination of the enzyme from the blood through the milk because some low phosphatase values were found in the plasma of heavily producing cows. Owen et al. (55) observed that an increase

in phosphatase paralleled the increase in chlorides when a quarter was affected by mastitis. On the other hand, Folley and Kay (20) concluded that milk phosphatase originates from the mammary gland and that it has probably no relation to the blood serum phosphatase. They suggested that a higher rate of phosphatase elimination by the mammary gland was associated with a relatively lower level of cellular efficiency.

There are a few reports in the literature which discuss the location of alkaline phosphatase in milk. Kay and Graham (39) believed it to exist either in the very thin layer which covers the fat globules or to be adsorbed onto the fat globules in such a way that the greater part of it may be removed by further treatment, e.g., churning. Sjostrom suggested the possibility of milk phosphatase linked with various fractions of the milk proteins as well as the lipids of the fat globule membrane. Hetrick and Tracy showed that the enzyme probably is concentrated at the fat serum interface. Rimpila and Palmer (56) made the observation that only 50% of the phosphatase in cream can be washed away and concluded that the remaining part of the enzyme is a constituent of the fat globule membrane and cannot be removed with water. Morton (53) showed that 30-40% of the phosphatase of milk is bound to the butterfat globules and is released

into the buttermilk on churning. He found that the phosphatase in fat-free separated milk and buttermilk is not in true solution in the serum but is associated with an insoluble particulate lipoprotein complex.

Little is definitely known about the chemical nature of the phosphatase in milk. Massart and Vandendrseissche (50) suggested that the enzyme is a metal protein complex in which a heavy metal like zinc is included rather than Mg^{++} or Mn^{++}. Anderson (3) assumed that this enzyme protein is a mixture of several components. According to Moelwin-Hughes (52) such components may be at different stages in their electrolytic dissociation.

Development of Methods for the Determination of Phosphatase Activity

It might be of interest to make brief mention here of methods of estimating phosphatase activity of blood serum, which can be considered as forerunners of today's determinations of milk phosphatase activity.

In 1925, phosphatases from blood serum and mammary gland of the cow were studied by Kay (36). Seven years later Wilson and Hart determined phosphatase activity in the milk as well as in the blood plasma of the cow using the

method of Kay (37). The same method, slightly modified, as for blood was used for the determination of milk phosphatase activity. The substrate was pure crystalline sodium β-glycerophosphate and the hydrolysis time 48 hours.

King and Armstrong (43) used disodium monophenyl phosphate as substrate for the determination of phosphatase activity of human serum. The phenol reagent of Folin and Ciocalteu was used for the color development. Bodansky (11) worked with sodium β-glycerophosphate as substrate in the determination of phosphatase activity of human serum. Ross et al. (58) chose the same substrate for the determination of intestinal phosphate activity in rats. Shinowara et al. (63) developed a micro method for the determination of serum phosphatase activity using sodium β-glycerophosphate. This method has been modified by Gould and Schwachman (23) and was applied by Madsen and Tuba (47) in their study on the source of alkaline phosphatase in rat serum and the role of the enzyme in intestinal absorption. Huggins and Talalay, Bessey et al. (9) and Seligman et al. (62) reported methods employing different substrates including phenolphthalein phosphate, p-nitrophenyl phosphate and sodium β-naphthyl phosphate.

In 1935, Kay and Graham (40) proposed their phosphatase test for pasteurized milk. Since that time several modified methods have been developed to measure the residual alkaline phosphatase activity in dairy products after pasteurization. Burgwald (13) reviewed the literature up to 1939 on the application of the phosphatase test for detecting irregularities in the pasteurization of milk and dairy products. In 1947, Sanders and Sager presented a more sensitive phosphatase test for pasteurization applicable to a variety of dairy products.

In 1949, Sjostrom reported a comparison of three phosphatase tests, viz., the Kay and Graham glycerophosphate method modified by Stein (68), the Kay and Graham phenyl phosphate method (40) and, finally, the modification of the latter by Scharer (60). Sjostrom abandoned the most sensitive method of Kay and Graham because the phenol reagent used in this method also reacts with certain products of protein decomposition. He proposed the Kay-Graham-Stein (K-G-S) test despite the fact that this test was less sensitive than Kay and Graham's phenyl phosphate method. In 1949, Aschaffenburg and Mullen (5) reported a rapid phosphatase test using disodium p-nitrophenyl phosphate as substrate yielding the yellow color of p-nitrophenol. The procedure of Sanders and Sager was adopted as an official method for

testing fluid milk by the Association of Official Agricultural Chemists (6) in 1950. The latest information on certain modified phosphatase tests was published by Scharer (61) in 1953.

Properties Involved in Estimation of Phosphatase Activity. Kinetics.

The estimation of the residual phosphatase is important in any phosphatase test for pasteurization. The amount of an enzyme may be measured by the results it produces, that is by its "activity". An accurate procedure for the determination of phosphatase activity should provide optimal conditions where all the factors which are known to influence enzyme activity are maintained constant, if possible. Many of the reports in the literature on milk phosphatase methods do not present data to show that the particular procedure meets the above requirements. Folley and Kay (19) determined certain kinetic data for the alkaline phosphomonoesterase of the mammary gland of guinea pigs. This information was undoubtedly valuable in developing the technique for the Kay and Graham phosphatase test for pasteurizing milk. Aschaffenburg and Mullen described experiments to determine

optimum conditions for their phosphatase test but similar data for the Sanders and Sager method have not been reported.

Methods for detecting phosphatase activity of milk have been mainly used to estimate the residual phosphatase in dairy products after pasteurization. It is surprising that, in spite of the importance of the phosphatase test, little fundamental information essential to the evaluation of the results obtained by the various techniques has been published.

Considerable attention has been paid to the heat inactivation of milk phosphatase by pasteurization. However, with the exception of the report of Kannan and Basu (35) on a phosphatase concentrate from Indian cow's milk, the literature contains no extensive studies on the kinetics of milk phosphatase.

EXPERIMENTAL

A. Alkaline Phosphatase Activity of Raw Milk

I. MATERIALS AND METHOD

(a) Sources of Milk

Individual milks were obtained from the University herd. The samples were taken from the morning yield, cooled to room temperature and examined for phosphatase activity within four hours. Table I gives information about the cows and the periods when their milks were tested.

Pooled raw milks were provided by three Edmonton dairy plants from 1500 gal. lots and were tested within 24 hours of sampling.

(b) Method and Apparatus

A modification of the method of Sanders and Sager was used to determine the phosphatase activity of raw milk. The activity was expressed as γ phenol released per 0.5 ml. milk under the described experimental conditions.

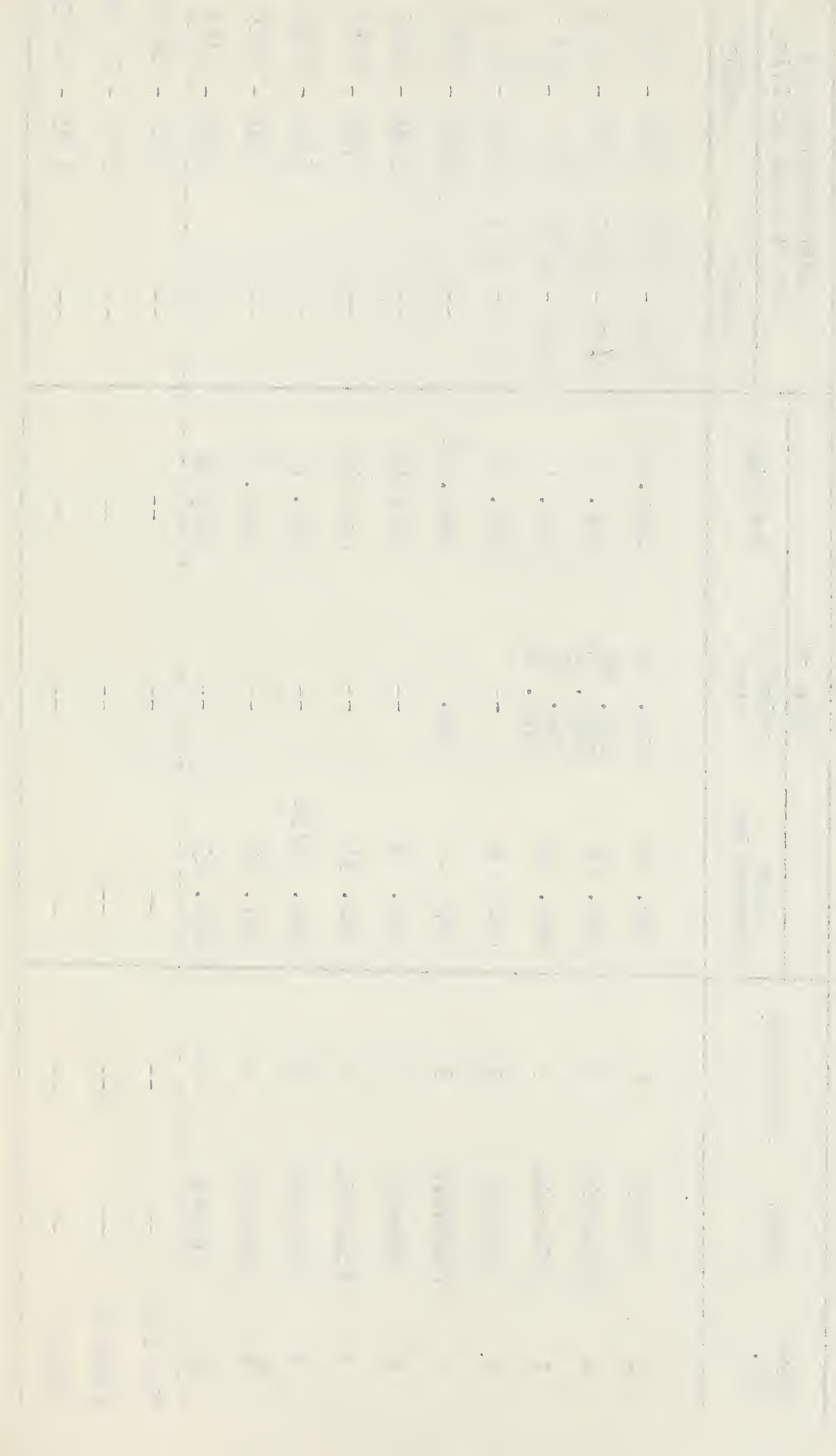
The water bath provided selected temperatures in the range 25 - 30°C. with an accuracy of $\pm$0.15°C.

Hydrogen ion concentrations were determined with a model G Beckman glass electrode pH meter standardized with Beckman buffer solutions.

TABLE I

RAW MILK SOURCES AND PERIODS OF TESTING

Cow No.	Breed	Lactation	Date of			Determination of phosphatase activity	
			Last parturition	Period of heat	Breeding	Daily	Weekly
1	Jersey	6	Mar. 18	Aug. 14	Sept. 4	5/20 - 6/18	5/26 - 8/25
2	Holstein	7	Mar. 21	Aug. 26 Sept. 17	Oct. 7	5/23 - 6/21	5/26 - 8/25
3	Holstein	5	Mar. 26	Aug. 19 Sept. 11	Oct. 4	5/28 - 6/26	6/ 2 - 9/ 1
4	Jersey	3	June 4	--	Oct. 2	6/ 6 - 7/ 5	6/ 9 - 9/ 8
5	Holstein	3	May 2	Aug. 2	Sept. 12	--	5/26 - 8/25
6	Jersey	5	Apr. 9	--	June 29	--	5/26 - 8/25
7	Holstein	4	Mar. 31	--	June 16	--	5/26 - 8/25
8	Holstein	4	Dec. 25/52	--	Apr. 4	--	5/26 - 8/25
9	Holstein	4	Apr. 23	--	Sept. 29	--	5/26 - 8/25
10	Jersey	5	Apr. 13	--	July 12	--	5/26 - 8/25
Plant A	--	--	--	--	--	--	5/27 - 12/23
Plant B	--	--	--	--	--	--	8/25 - 12/23
Plant C	--	--	--	--	--	--	8/25 - 12/23



An Evelyn photoelectric colorimeter was used for measuring the transmittance at approximately 600 mμ in the determination of phenol.

A Beckman quartz spectrophotometer, model DU, was used for spectrophotometric work.

The glassware used in this investigation was first soaked and washed in detergent, then placed in a 5% nitric acid bath for several hours and finally was rinsed and soaked in distilled water.

II. RESULTS AND DISCUSSION

(a) Modification of Sanders and Sager Method

Unpublished results of Hansen suggested that the method of Sanders and Sager does not provide optimal experimental conditions for the estimation of phosphatase activity in raw milk. Therefore, preliminary studies of various steps in their procedure were made.

1) Hydrolysis time. Evidence was obtained that the phosphatase activity measured after 30 minutes of hydrolysis and multiplied by two was consistently greater than when measured after one hour, as specified in the Sanders and Sager technique.

2) Color development. Development of the indophenol blue color at 25°C. for 30 minutes gave more uniform results than the variable "room temperature" suggested by Sanders and Sager.

3) Dilution procedure. Sanders and Sager recommended that strongly positive tests be diluted one or more times, more indicator added and 30 minutes allowed for color development after each dilution. In the present investigation, the number of dilution steps was reduced and the possibility of production of off-colors by deterioration of the indicator during color development was diminished.

4) Calibration curve. When the sequence suggested by Sanders and Sager was followed in the preparation of the phenol standards, a flocculent precipitate was formed. It was found that this precipitate could be avoided by mixing the reagents in the following sequence:

(1) 0.05% copper sulphate solution /2/
(2) distilled water /4/
(3) dibromoquinonechloroimine (B.Q.C.) /5/
(4) color dilution buffer /3/
(5) phenol /1/

/-/ = sequence proposed by Sanders and Sager

Fig. 1 shows the absorption curve for indophenol blue in aqueous solution. The filter used in the present

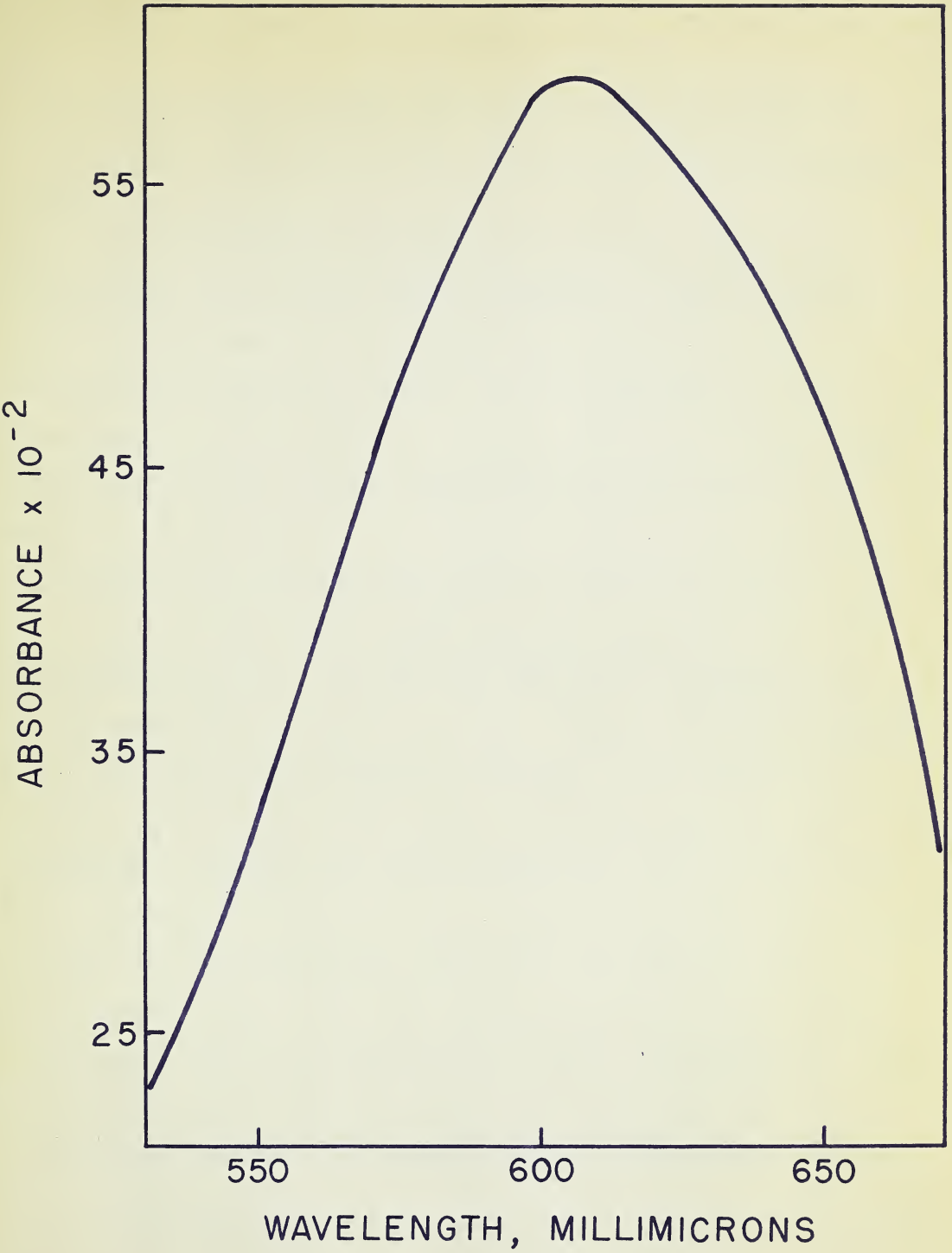


Fig. 1. Absorption maximum for colorimetric estimation of phenol in determination of phosphatase activity.

investigation had maximum light transmission in the 600 mμ region which falls within the absorption maximum of indophenol blue.

The calibration curve for the Sanders and Sager method is obtained from aqueous phenol standards. These standards are not prepared under the conditions of the test with regard to the presence of milk, buffer substrate, protein precipitant or the influence of heat. Therefore, experiments were carried out in which phosphatase determinations were made on raw and pasteurized milks containing known amounts of phenol with the elimination of the hydrolysis period.

Fig. 2 compares the usual standard curve with the one obtained with milk. The results show that only part of the phenol added to milk was recovered. A question arises whether the same proportion of phenol liberated by the enzyme is always found in the determination of phosphatase activity. Although a straight-line relationship was found in the above experiments, the data are too limited to establish this point. If the phosphatase activities presented in this thesis were based on the calibration curve with milk, the data would have to be multiplied by 1.91. To facilitate comparison with other reports in the literature, the calibration curve prepared from aqueous phenol standards was employed.

Fig. 2. Calibration curves prepared with standard amounts of phenol:
A - in water, M - in milk.

5) Summary of modified method. A modified Sanders and Sager method was adopted in which the same reagents were used but certain steps in the procedure were changed.

The hydrolysis time was shortened to 30 minutes and, following precipitation and filtration steps, 0.5 ml. filtrate was made up to 10 ml. with color development buffer. Four drops (0.08 ml.) of 2,6-dibromoquinonechloroimine solution (B.Q.C.) were added and further dilution (final dilution 1:200 in most cases), accompanied by addition of two drops of B.Q.C., was made. The color was developed at 25°C. for 30 minutes. Phosphatase activity was expressed in terms of γ phenol released by the enzyme contained in 0.5 ml. milk under the above conditions, calculated for a hydrolysis time of one hour.

(b) Phosphatase Activity of Milks from Individual Cows

1) Variation between milks. Table II shows the variation of phosphatase activity between the milks of 10 individual cows in different stages of lactation. Information on the samples is provided in Table I. At the beginning of the three-month period of determinations, the levels of phosphatase activity ranged from 100 to 500 and at three months from 550 to 1450 γ phenol. These results indicate that the extent of variation between individual cows varied widely.

TABLE II

VARIATION OF PHOSPHATASE ACTIVITY BETWEEN MILKS OF TEN INDIVIDUAL COWS IN DIFFERENT STAGES OF LACTATION AND CORRESPONDING MILK PRODUCTION DURING JUNE, JULY AND AUGUST, 1953

Cow No.	Weekly determinations of phosphatase activity* and corresponding milk production**													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	149 (27.6)	149 (30.0)	160 (29.0)	291 (27.0)	276 (27.2)	288 (25.5)	342 (24.9)	360 (18.0)	422 (21.7)	516 (21.6)	394 (18.0)	624 (22.4)	740 (18.6)	742 (17.4)
2	416 (34.0)	176 (44.5)	178 (42.3)	223 (35.3)	321 (28.5)	308 (30.2)	323 (30.0)	258 (30.0)	360 (32.1)	787 (33.5)	557 (31.0)	547 (33.3)	835 (26.0)	931 (26.0)
3	119 (25.1)	121 (31.6)	245 (33.8)	232 (35.0)	159 (30.0)	350 (35.2)	389 (34.7)	547 (35.5)	624 (35.2)	451 (36.5)	544 (36.4)	682 (31.6)	806 (30.0)	840 (30.0)
4	110 (20.0)	100 (21.7)	135 (20.0)	239 (20.0)	304 (19.7)	321 (17.2)	490 (19.4)	499 (18.1)	470 (16.0)	557 (15.4)	998 (14.3)	991 (14.7)	1162 (17.3)	990 (17.0)
5	106 (38.0)	102 (40.0)	128 (37.7)	163 (35.5)	144 (35.2)	136 (36.4)	149 (35.4)	167 (33.2)	199 (28.5)	257 (22.1)	300 (19.0)	372 (17.0)	679 (17.5)	557 (22.7)
6	427 (20.7)	450 (22.4)	485 (21.3)	656 (21.2)	626 (21.0)	714 (19.8)	714 (19.5)	787 (20.2)	1061 (19.2)	1075 (19.2)	1090 (19.5)	1187 (19.5)	1411 (15.0)	1440 (16.4)
7	494 (28.0)	350 (32.5)	379 (31.2)	557 (30.4)	480 (28.0)	475 (24.0)	528 (25.5)	581 (26.0)	846 (27.0)	852 (25.5)	746 (21.0)	926 (23.5)	1192 (20.3)	1224 (18.5)
8	458 (25.4)	462 (26.7)	457 (26.4)	643 (24.8)	457 (24.1)	468 (23.0)	538 (23.2)	706 (23.0)	692 (22.0)	746 (20.0)	806 (20.5)	681 (20.4)	893 (19.0)	946 (19.6)
9	149 (33.4)	131 (36.3)	193 (30.0)	243 (33.0)	187 (29.6)	190 (32.5)	214 (31.5)	263 (30.6)	258 (31.4)	312 (30.8)	336 (30.0)	372 (29.5)	475 (27.5)	557 (24.0)
10	150 (18.2)	157 (10.0)	188 (19.0)	340 (17.0)	337 (16.0)	339 (16.0)	330 (14.6)	312 (14.3)	360 (14.5)	422 (14.6)	530 (13.2)	521 (12.0)	514 (9.8)	550 (11.7)

*All phosphatase activities reported in this thesis are expressed as γ phenol.

**All milk production data reported in this thesis are given in (pounds).

Samples from cows 5, 6, 7 and 10 showed a sharp rise in phosphatase activity in the 13th, 9th, 9th and 11th week, respectively. According to Table I, this rise occurred within three to five weeks after the cows had been served by the bull for the last time, i.e., after conception. Folley and Kay (20) also observed fluctuations near the time when the cow is in heat and has been served by the bull. It seems to be evident that disturbances in the physiological condition of the animal, such as pregnancy and sexual excitement, have an influence upon the phosphatase activity of the milk.

2) Effect of stage of lactation, milk yield and season. The phosphatase activities of four individual raw milks, obtained during the first 30 days after parturition, are shown in Table III. The first milking had a comparatively high activity which rapidly dropped to a low minimum after approximately one week to 10 days. This rapid decrease in phosphatase activity was followed by a gradual increase which is illustrated in Fig. 3. There is no relationship between phosphatase activity and milk yield during the first 30 days after parturition. It is obvious that the results obtained during the first few days after delivery of the calf represent another example of the pronounced effect of disturbances in the physiological condition of the animal

TABLE III

VARIATION OF PHOSPHATASE ACTIVITY DURING THE THIRTY DAYS[A] AFTER PARTURITION

Cow No.	Daily determinations of phosphatase activity and corresponding milk production														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	490[a] (6.7)	389 (25.8)	163 (23.0)	139 (23.0)	134 (24.8)	151 (26.3)	149 (27.6)	158 (27.5)	125 (29.4)	142 (27.4)	147 (28.6)	152 (29.3)	145 (29.8)	149 (30.0)	146 (29.4)
2	1733[b] (25.8)	1112 (29.0)	547 (29.0)	416 (34.0)	278 (39.0)	154 (38.0)	161 (40.0)	165 (43.6)	132 (42.4)	155 (38.6)	177 (44.5)	174 (45.5)	179 (44.0)	174 (40.0)	178 (41.8)
3	653[c] (16.7)	528 (16.7)	442 (19.0)	155 (20.4)	216 (23.5)	119 (25.1)	111 (23.5)	103 (28.5)	107 (28.1)	113 (22.6)	115 (31.0)	132 (30.6)	121 (31.6)	129 (31.5)	136 (30.5)
4	509[d] (14.0)	157 (16.8)	116 (20.6)	110 (20.0)	61 (18.4)	72 (20.2)	76 (19.8)	63 (20.0)	76 (22.4)	85 (21.8)	100 (21.7)	109 (21.4)	107 (22.3)	128 (21.5)	111 (21.9)

Cow No.	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	150 (30.0)	122 (30.0)	155 (31.0)	159 (31.4)	155 (28.0)	160 (29.0)	154 (27.3)	159 (26.7)	175 (28.4)	190 (26.3)	216 (27.0)	266 (26.4)	291 (27.0)	244 (25.5)	253 (26.7)
2	180 (42.4)	184 (40.0)	178 (42.3)	166 (40.0)	160 (40.0)	191 (40.0)	231 (37.2)	226 (38.4)	208 (37.4)	223 (35.3)	250 (36.0)	252 (33.0)	250 (33.3)	222 (33.0)	234 (28.0)
3	180 (32.8)	217 (34.0)	221 (33.5)	228 (33.5)	245 (33.8)	224 (33.5)	253 (34.3)	259 (38.0)	199 (41.5)	207 (39.5)	210 (39.0)	232 (35.0)	231 (35.0)	298 (34.5)	269 (35.4)
4	122 (20.0)	140 (21.6)	135 (20.0)	273 (18.7)	241 (17.8)	272 (18.0)	281 (18.4)	214 (19.2)	204 (18.4)	239 (20.0)	250 (18.4)	258 (20.3)	248 (21.6)	243 (20.4)	245 (19.5)

[A] May 20 to July 5, 1953.

[a] 3rd milking
[b] 1st milking
[c] 4th milking
[d] 2nd milking

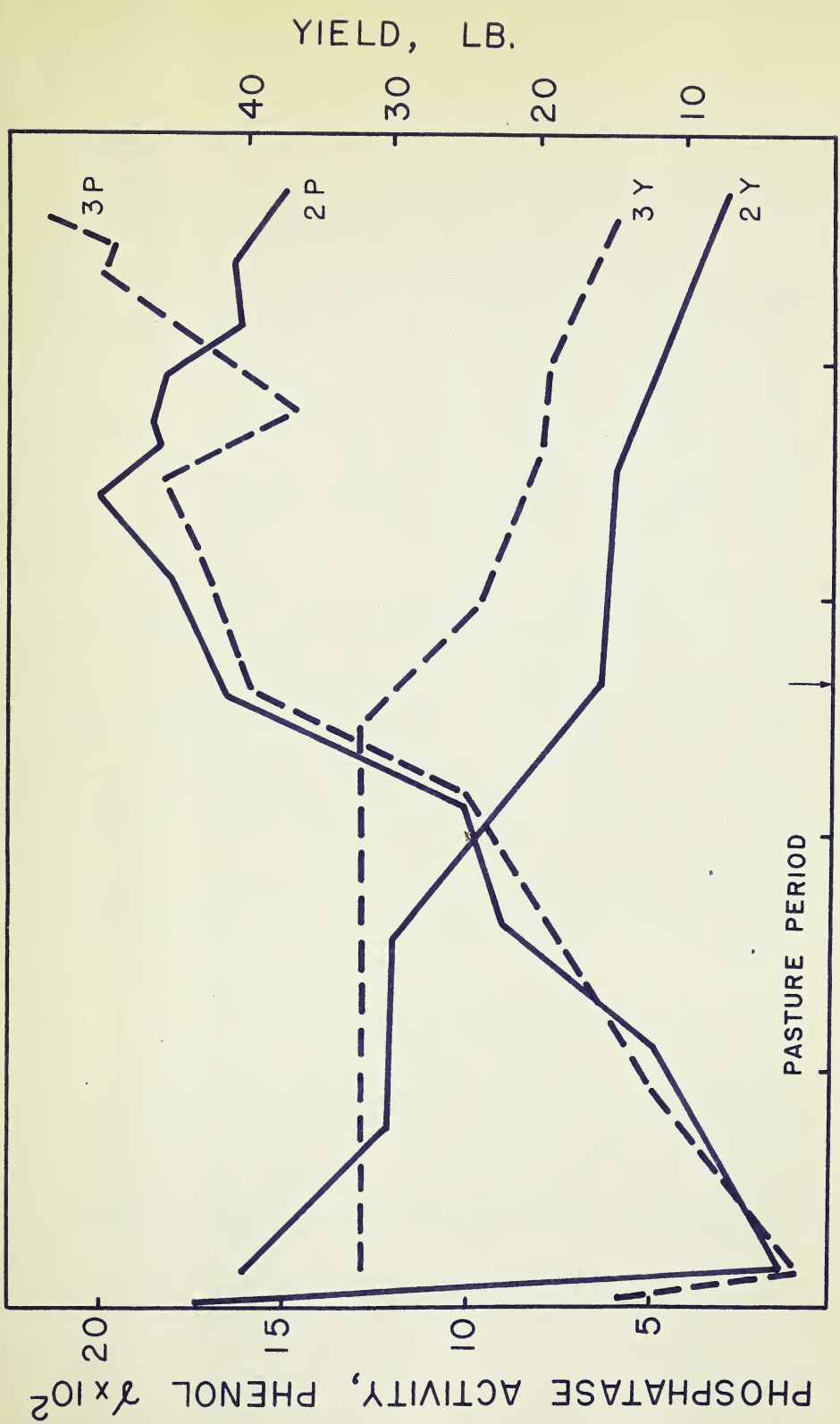


Fig. 3. Phosphatase activity (P) and milk yield (Y) for cows No. 2 and No. 3.

upon the phosphatase content of milk. Folley and Kay (20) reported that the phosphatase activity dropped to a minimum between 10 and 25 days after parturition.

Table IV summarizes phosphatase activity determinations on the milks of four cows, which calved within the same two weeks, during the first 40 to 42 weeks of the lactation period. The results ranged from 76 to 4378 γ phenol. There are no comparable data obtained by the Sanders and Sager method reported in the literature. However, Folley and Kay (20) and Kannan and Basu also found variations in the activities of individual milks using different methods.

Fig. 3 illustrates the variation of enzyme activity in the milks of cows 2 and 3. The curves show a peak about seven months after parturition. The data in Table IV indicate that the milks of cows 1 and 4 also possessed maximum phosphatase activity around seven months after parturition. This is about one month later than reported by Folley and Kay (20).

Fig. 3 shows that the phosphatase activity increased after 7 to 10 days following parturition until the seventh month, but the milk yield decreased after the third and fifth month for cows 2 and 3 respectively. The data in Table IV indicate that the phosphatase activity of milks from

TABLE IV

EFFECT OF STAGE OF LACTATION AND SEASON ON PHOSPHATASE ACTIVITY DURING ONE LACTATION PERIOD

Cow No.	Phosphatase activity and corresponding milk production																			
	Number of weeks after parturition																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	134 (24.8)	145 (29.8)	159 (31.4)	216 (27.0)	276 (27.2)	288 (25.5)	342 (24.9)	360 (18.0)	422 (21.7)	516 (21.6)	394 (18.0)	624 (22.4)	746 (18.6)	742 (17.4)	910 (18.5)	970 (16.5)	--	--	1201 (11.0)	1188 (11.0)	1570 (3.5)
2	161 (40.0)	174 (40.0)	191 (40.0)	250 (33.3)	321 (28.5)	308 (30.2)	323 (30.0)	258 (30.0)	360 (32.1)	787 (33.5)	557 (31.0)	547 (33.3)	835 (26.0)	931 (26.0)	979 (24.7)	893 (23.5)	--	--	998 (18.4)	1104 (16.4)	1008 (17.6)
3	119 (28.1)	121 (31.6)	245 (33.8)	232 (35.0)	259 (30.0)	350 (35.2)	389 (34.7)	547 (35.5)	624 (35.2)	541 (36.5)	544 (36.4)	682 (31.6)	806 (30.0)	840 (30.0)	667 (33.0)	--	--	1051 (27.4)	1181 (25.1)	1162 (23.0)	1291 (20.7)
4	76 (19.8)	128 (21.5)	272 (18.0)	248 (21.6)	304 (19.7)	321 (17.2)	490 (19.4)	499 (18.1)	470 (16.0)	557 (15.4)	998 (14.3)	991 (14.7)	1162 (17.3)	990 (17.0)	--	--	1234 (7.6)	1354 (8.6)	1411 (6.3)	2959 (6.2)	3029 (7.0)
	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
1	1621 (10.0)	1565 (7.4)	1954 (8.3)	1824 (6.5)	1973 (6.8)	2544 (7.4)	2467 (2.0)	1728 (7.6)	2026 (7.0)	1584 (4.2)	1272 (3.0)	2362 (6.4)	2323 (7.5)	2362 (7.1)	2064 (7.2)	2136 (6.3)	2448 (7.0)	2000 (7.6)	2131 (6.5)	1997 (6.7)	1958 (7.0)
2	1090 (15.5)	1410 (15.2)	1709 (16.2)	1757 (12.8)	1997 (12.3)	1723 (12.3)	1838 (9.1)	1939 (9.7)	2021 (10.2)	1810 (10.5)	1824 (10.5)	1901 (9.8)	1526 (10.5)	1877 (9.1)	1368 (7.0)	1440 (6.5)	1478 (8.0)	1632 (8.0)	1628 (6.5)	1424 (6.0)	1315 (6.4)
3	1642 (21.5)	1445 (20.0)	1632 (19.8)	1730 (19.0)	1594 (18.8)	1649 (20.1)	1595 (15.8)	1829 (20.2)	1742 (19.0)	1815 (19.0)	1800 (18.0)	1405 (19.0)	1421 (18.1)	1540 (14.3)	1430 (17.2)	2030 (16.8)	1987 (16.7)	1910 (12.6)	2016 (12.3)	2323 (12.3)	--
4	3552 (7.3)	2354 (7.3)	2986 (6.0)	3744 (7.0)	3907 (7.0)	3648 (7.2)	4378 (7.2)	4272 (7.2)	4224 (7.0)	3034 (6.2)	3120 (6.8)	3178 (6.7)	3288 (6.5)	3355 (6.4)	3331 (6.5)	3336 (7.0)	3336 (6.6)	3264 (6.1)	3086 (6.0)	--	--

cows 1, 2 and 4 decreased after reaching a maximum around the seventh month after parturition. However, the milk from cow 3 did not follow this trend. Folley and Kay (20) pointed out that the milk yield - lactation curve was almost the reverse of the phosphatase - lactation curve.

Table IV and Fig. 3 and 4 suggest that the change from summer to winter feeding did not result in unusual variations in phosphatase activity.

(c) Phosphatase Activity of Pooled Milks

1) Variation between milks. The results obtained during four months for pooled milks from three different plants receiving milk from the same area are shown in Table V. There was little variation between the milk obtained from the three plants and determinations for plants B and C were discontinued after the fourth month. Furthermore, fluctuations in phosphatase activity did not appear to be related to variations in fat content. This finding is in agreement with the report of Storrs and Burgwald (70).

Fig. 4 shows that the activities for the pooled milks from plant A varied from 950 to 1700 γ phenol during the period May 27, 1953 to March 15, 1954. The few determinations on pooled milks reported by Hetrick and Tracy and Sanders and Sager fall within this range.

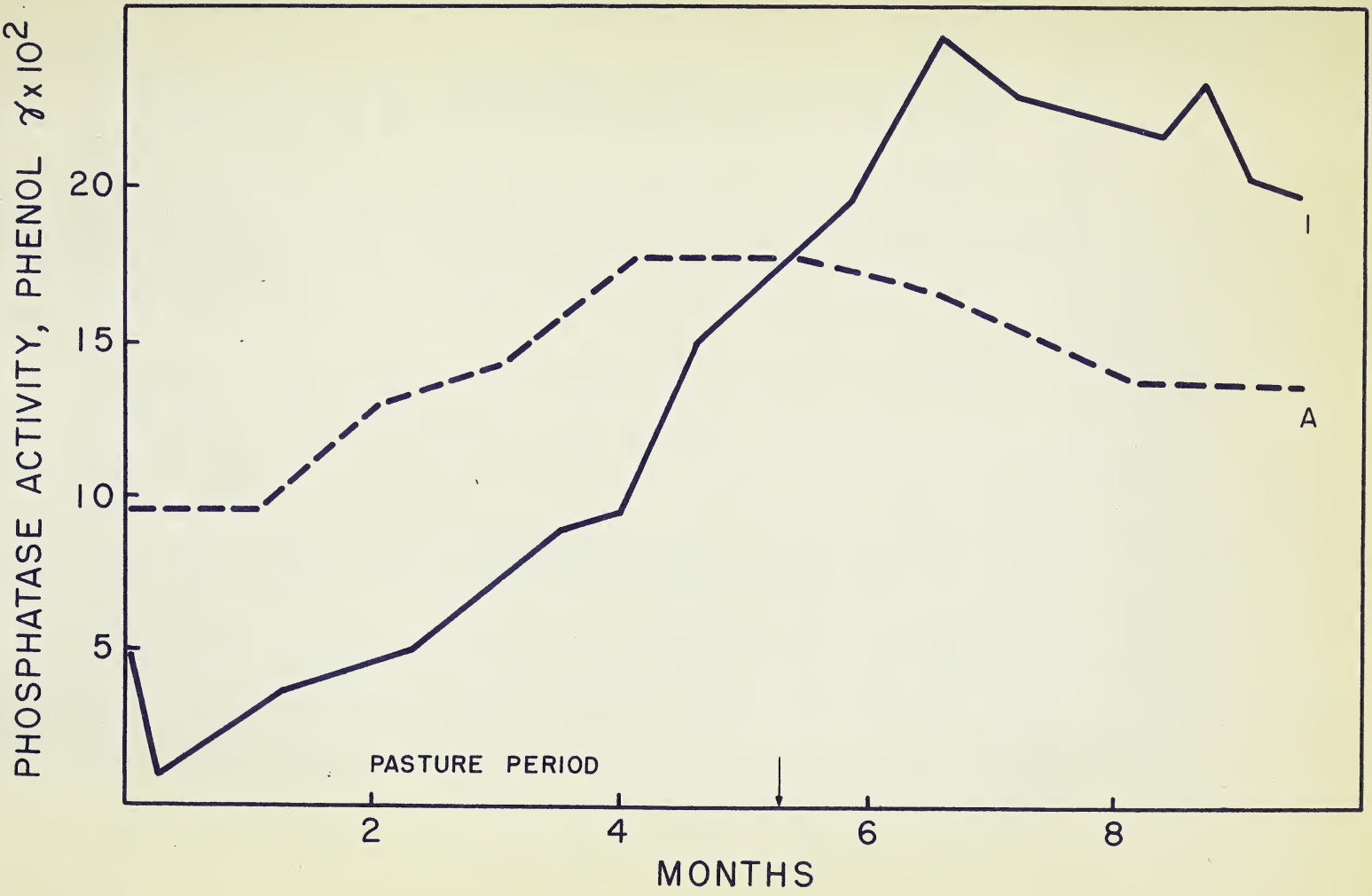


Fig. 4. Phosphatase activity of individual milk (cow No. 1) and of pooled milk (plant A).

TABLE V

VARIATION IN PHOSPHATASE ACTIVITY AND FAT PERCENTAGE OF POOLED MILK FROM THREE EDMONTON DAIRY PLANTS DURING FOUR MONTHS

Date of Determination	Phosphatase activity and corresponding fat percentage					
	Plant					
	A		B		C	
8/25	1474	(3.55)	1493	(3.50)	1430	(3.55)
9/ 2	1454	(3.55)	1478	(3.50)	1454	(3.55)
9/ 9	1594	(3.55)	1570	(3.45)	1550	(3.50)
9/30	1699	(3.50)	1518	(3.50)	1685	(3.45)
10/ 7	1685	(3.50)	1750	(3.45)	1507	(3.55)
10/14	1580	(3.45)	1458	(3.50)	1632	(3.50)
10/21	1684	(3.55)	1411	(3.50)	1651	(3.45)
10/28	1622	(3.50)	1554	(3.45)	1649	(3.50)
11/ 4	1570	(3.50)	1411	(3.50)	1430	(3.45)
11/11	1477	(3.50)	1550	(3.50)	1738	(3.50)
11/18	1730	(3.50)	1554	(3.45)	1536	(3.45)
11/25	1555	(3.45)	1594	(3.40)	1512	(3.45)
12/ 2	1545	(3.50)	1635	(3.50)	1595	(3.45)
12/ 9	1515	(3.45)	1660	(3.40)	1430	(3.40)
12/16	1550	(3.40)	1532	(3.45)	1550	(3.40)
12/23	1570	(3.40)	1415	(3.55)	1553	(3.50)

2) Effect of stage of lactation and season. Fig. 4 compares the phosphatase activities of milks from cow 1 and plant A. The flat peak of the pooled milk curve is explained by the fact that the individual milks do not display their maximum activity at exactly the same time. Cow 1 calved about one month later than most animals in the Edmonton area and this may account for the shift in maxima in Fig. 4.

Seasonal feeding changes did not result in marked fluctuations of phosphatase activity of the pooled milk.

B. Properties Involved in Estimation of Phosphatase Activity. Kinetics

I. MATERIALS AND METHOD

Individual milks were obtained from the University herd and pooled raw milks from plant A.

The apparatus employed and the procedure used to clean the glassware have been described in section A.

Phosphatase activities were expressed as either γ phenol or γ phosphorus released by the enzyme contained in 0.5 ml. milk under the described experimental conditions. The procedure for the estimation of phosphatase activity using disodium phenyl phosphate has been summarized in section A. II. (a). Where the substrate was sodium β-glycerophosphate, phosphorus was determined colorimetrically by a modification of the method of Gould and Schwachman.

II. RESULTS AND DISCUSSION

(a) pH of Catalyzed Reaction and Substrate Concentration

1) pH optimum. A series of buffers was prepared that gave a range of pH from approximately pH 8 to pH 12 when used in the phosphatase determinations. These buffers contained

varying proportions of boric acid and barium hydroxide and their composition is summarized in the appendix (Table B). The pH values of the reaction mixtures were determined initially and after 10, 20 and 30 minutes of the hydrolysis period. The average drop during hydrolysis of the substrate was not greater than 0.03 pH.

Fig. 5 shows the apparent pH optima of the hydrolysis of disodium phenyl phosphate catalyzed by the phosphatase of a pooled milk and the milk and colostrum, respectively, from an individual cow. The peaks of all three curves are at pH 10.0. On the more alkaline side of this optimum, the activity dropped more rapidly than on the less alkaline side. The curves show the importance of accurate pH adjustment in the determination of phosphatase activity, especially for colostrum.

In 1934, Graham and Kay (39) recommended a pH optimum of 9.2 for their determination of milk phosphatase. Results obtained above are in agreement with the pH employed in the Sanders and Sager method for raw milk.

2) Optimum substrate concentration. Hansen gave evidence that the 0.00834 M concentration of disodium phenol phosphate in the hydrolysis mixture suggested by Sanders and Sager did not saturate the amount of phosphatase in raw milk. Therefore, it was necessary to increase the substrate concentration.

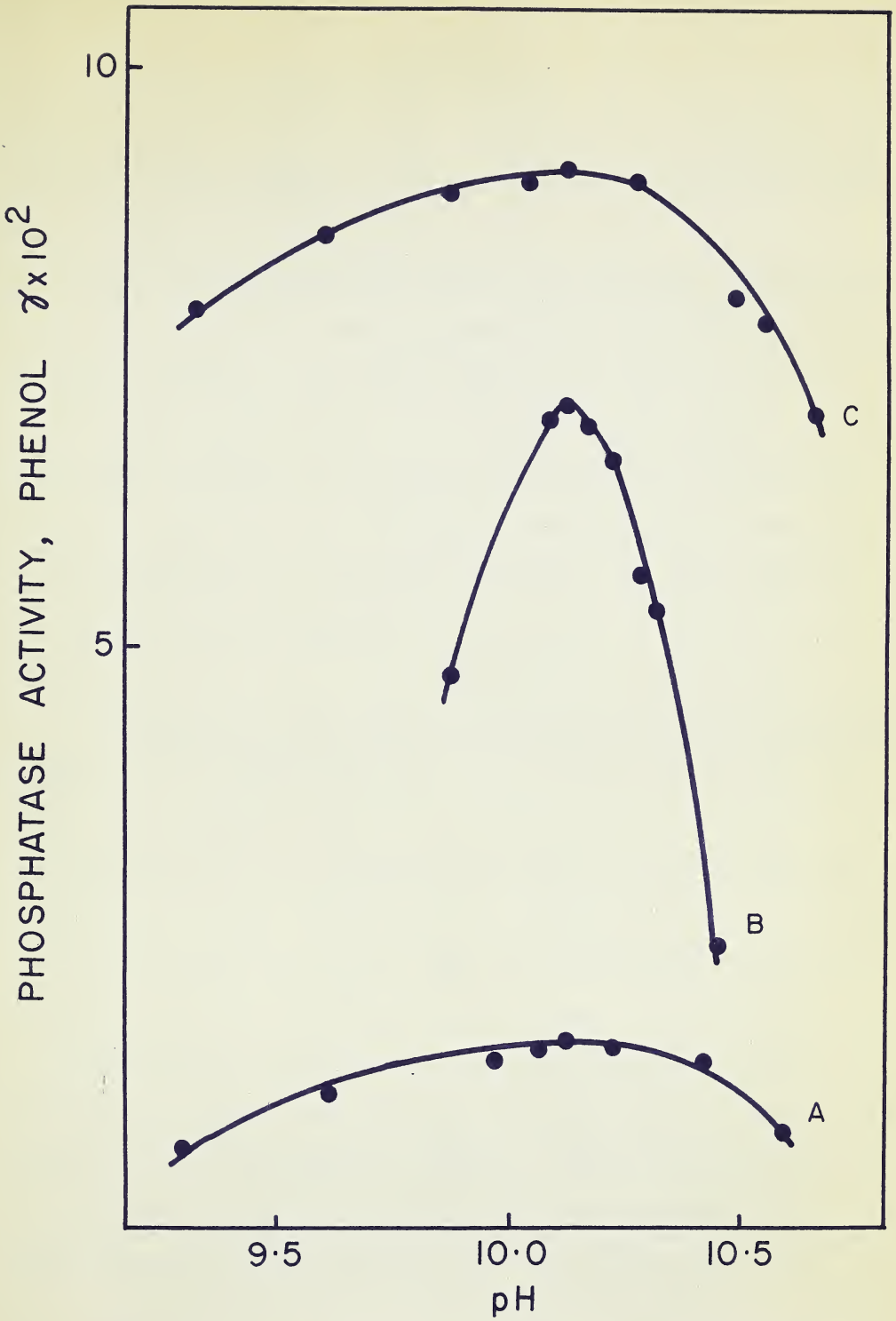


Fig. 5. Apparent pH optima of the hydrolysis of disodium phenyl phosphate by milk (A) and colostrum (B) from cow No. 2, and by pooled milk (C).

In the present investigation, determinations of phosphatase activity using different substrate concentrations were made at pH 10.0.

Fig. 6 illustrates the effect of substrate concentration upon the activity of alkaline phosphatase of individual and pooled raw milks. The enzyme appeared to be saturated at a substrate concentration of 0.02919 M sodium phenyl phosphate. A further increase in substrate concentration resulted in decreased activity. This might be partially attributed to inhibitory effect of reaction products. The data confirm the observation of Hansen.

3) Interdependence of pH optimum and substrate concentrations. It is recognized that the so-called "pH-curve" is not a true characteristic of an enzyme, but is a function of the stability of the enzyme protein to hydrogen ion concentration in the absence of substrate and the effect of pH on the catalyzed reaction. Thus the pH optimum depends on various factors, including the substrate concentration.

Therefore, subsequent to the determination of the optimum substrate concentration, the pH optimum of the catalyzed reaction was redetermined. Table VI shows the variation in phosphatase activity of an individual and a pooled milk where the pH of the catalyzed reaction varied and three

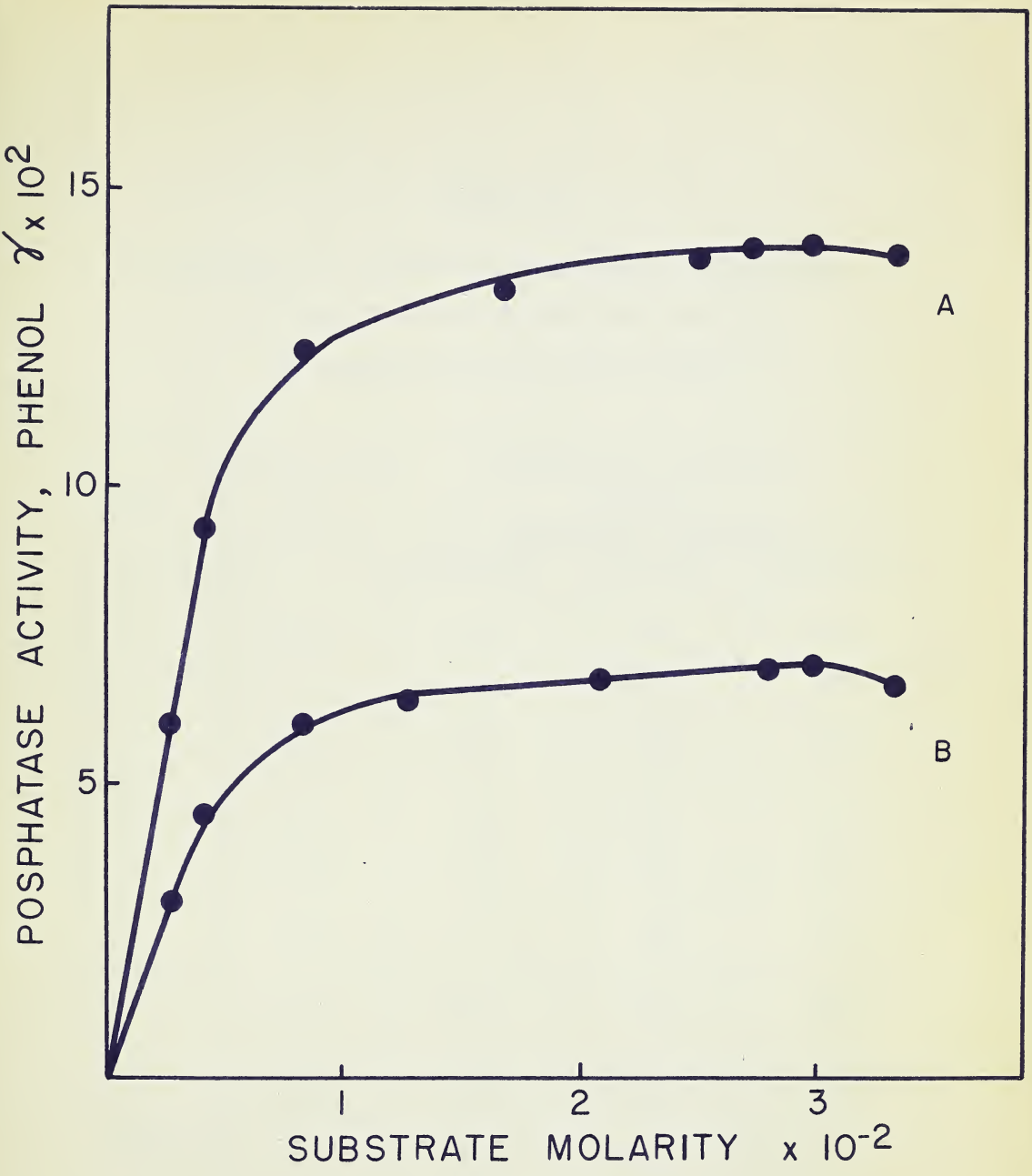


Fig. 6. Effect of increasing substrate concentration upon phosphatase activity of pooled (A) and individual (B) milks.

TABLE VI

INFLUENCE OF AVERAGE REACTION pH AND SUBSTRATE CONCENTRATION ON PHOSPHATASE ACTIVITY OF INDIVIDUAL AND POOLED MILKS

Milk Source	Average pH of reaction	Phosphatase activity, γ phenol		
		Substrate molarity		
		0.025020	0.029190	0.033230
Cow 5	10.02	223	263	183
	10.10	246	273	201
	10.17	272	288	220
	10.27	289	291	239
	10.33	272	297	261
	10.38	255	288	281
	10.52	-	261	253
Plant A	10.00	1034	1286	874
	10.18	1262	1329	1258
	10.27	1313	1339	1277
	10.32	1243	1392	1301
	10.39	1166	1349	1330
	10.47	1042	1224	1300

different substrate concentrations were employed. The new optimum pH value for the optimum substrate concentration was found to be 10.32 ±0.04. From these results it is obvious that changes in substrate concentration cause a shift of the pH optimum of the catalyzed reaction.

A number of experiments were carried out in order to investigate further the relation between pH optimum and substrate concentration. Table VII summarizes pH optima and maximum phosphatase activities of catalyzed reactions with various substrate concentrations. The results show that the phosphatase reactions with the different milks have the same optimum substrate concentration and the same pH optimum. Fig. 7 indicates that maximum hydrolysis of the substrate only occurs within a relatively narrow pH zone. From the results shown in Table VII and Fig. 7 it appears that at 37.5°C. the optimum hydrolysis of disodium phenyl phosphate by alkaline phosphatase of milk is defined by 0.02919 M substrate and pH 10.32. In addition, the results show that with increasing substrate concentration the corresponding pH optimum also increases. Similar observations were made by Folley and Kay (19) with alkaline phosphatase of mammary gland tissue of guinea pigs. Ross et al. also observed this shift of pH optima in a preparation of alkaline phosphatase from an extract of intestinal mucosa of rats.

TABLE VII

RELATION BETWEEN SUBSTRATE CONCENTRATION, pH OPTIMUM AND PHOSPHATASE ACTIVITY

Substrate molarity	pH optimum	Maximum phosphatase activity, γ phenol			
		Milk source			
		Cow 3	Cow 5	Cow 6	Plant A
0.002084	9.74	--	91	--	475
0.004165	9.76	--	185	--	855
0.008340	10.01	542	254	1095	1094
0.016680	10.10	677	272	1258	1215
0.025020	10.25	737	289	1381	1313
0.029190	10.32	780	297	1480	1392
0.033230	10.38	672	281	1305	1329

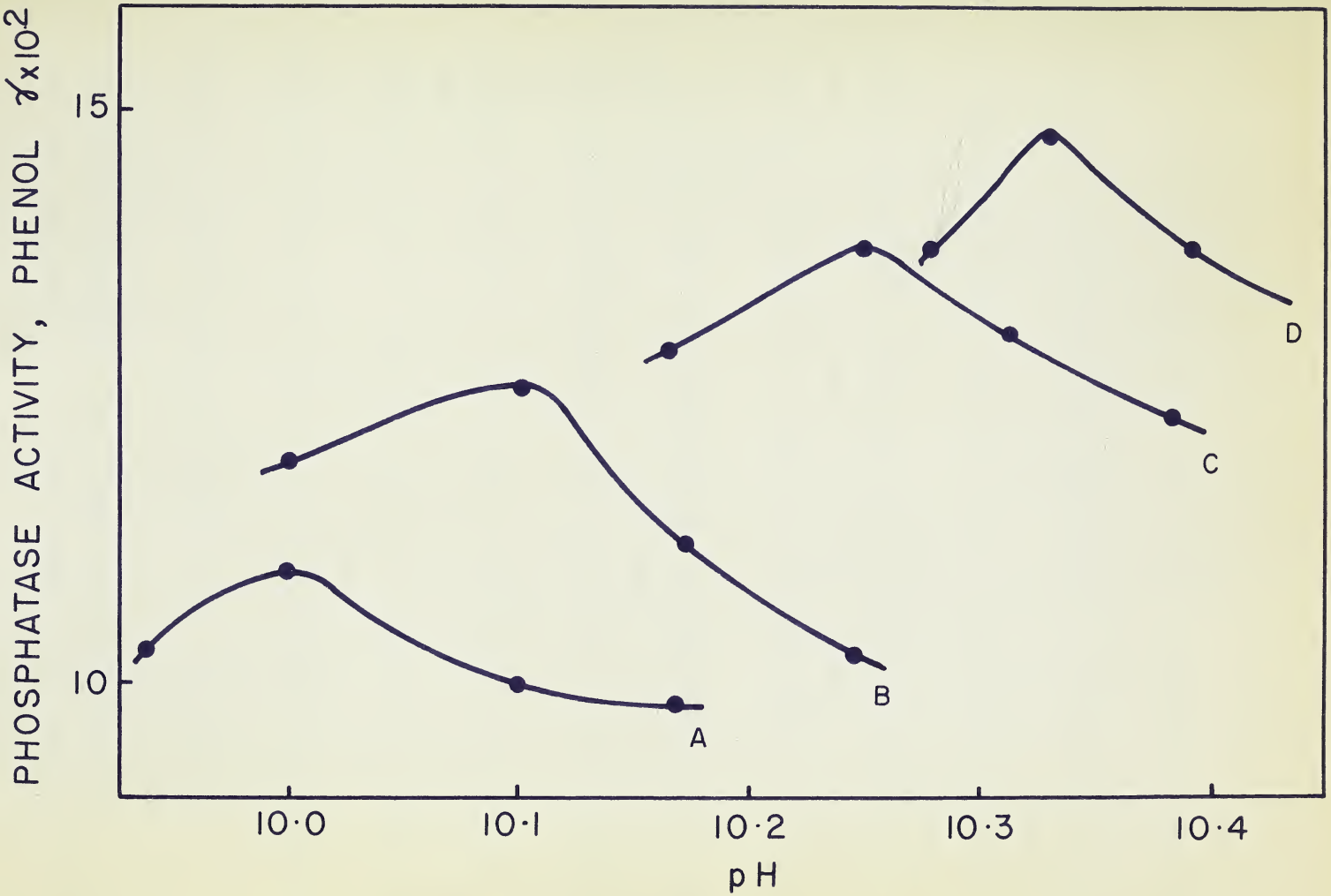


Fig. 7. Shift of pH optimum with increasing concentrations of substrate in the reaction mixture.

A - 0.008340 M, B - 0.016680 M, C - 0.025020 M, D - 0.029190 M.

(b) Optimum Hydrolysis Temperature

In order to determine the optimum experimental conditions for phosphatase activity, certain other factors including the effect of temperature, time of reaction and activation were studied. Preliminary experiments showed that the addition of magnesium sulfate did not result in increased substrate hydrolysis. Therefore, it was assumed that the magnesium ion concentration of milk was not a limiting factor in determination of phosphatase activity. Optimum reaction time and activation and inhibition are discussed later on in this thesis.

It has been generally observed by numerous workers that most enzymes act best in a fairly narrow temperature range. This temperature optimum can be a function of two variables: the temperature stability of the protein moiety of the enzyme and the temperature coefficient of the catalyzed reaction involved. If we consider the protein moiety to play an essential role in the function of an enzyme, the variability of temperature stability of the apoenzyme in absence of substrate can depend on the overall structure of the protein, on the type of the functional group or groups, and on the location of these essential groups, i.e., the specific configuration of the active centers. In addition, the temperature stability of an enzyme in the presence of substrate is affected by the formation of the complex between

the enzyme and the substrate. With regard to the influence of the temperature coefficient, it is known that an increase in temperature results in acceleration of a chemical reaction. The increase in rate for a 10°C. change can be described by the symbol Q_{10}.

Four different raw milks were used in the determinations of the optimum reaction temperature which were carried out at seven different temperatures ranging from 30 to 50°C. Two different substrate concentrations and their corresponding pH optima were used.

From the results summarized in Table VIII it can be seen that there was a slight increase in activity when the reaction temperature was raised from 37.5 to 38.5°C. The experiment with different substrate concentrations gave the same temperature optimum. Fig. 8 illustrates the activity change with the two different substrate concentrations at the various temperatures for the pooled milk. The activity decreased faster at temperatures above 38.5°C. than below. Relatively high activity was observed at a temperature of 50°C. The hydrolysis curve for the higher substrate concentration shows a relatively sharp peak compared to the curve obtained with the lower concentration.

TABLE VIII

TEMPERATURE OPTIMUM FOR THE HYDROLYSIS OF DISODIUM PHENYL PHOSPHATE BY PHOSPHATASE

Substrate molarity	Temperature °C.	Phosphatase activity, γ phenol			
		Milk source			
		Cow 3	Cow 5	Cow 6	Plant A
0.008340	30.0	424	222	1053	1132
	35.5	516	322	1162	1401
	37.5	542	354	1188	1435
	38.5	576	360	1214	1464
	40.0	552	312	1166	1420
	41.5	526	281	1180	1382
	50.0	379	270	949	840
0.029190	30.0	561	360	1232	1557
	35.5	662	401	1459	1752
	37.5	678	429	1458	1798
	38.5	706	473	1522	1858
	40.0	684	399	1478	1740
	41.5	667	354	1411	1550
	50.0	459	263	1013	1300

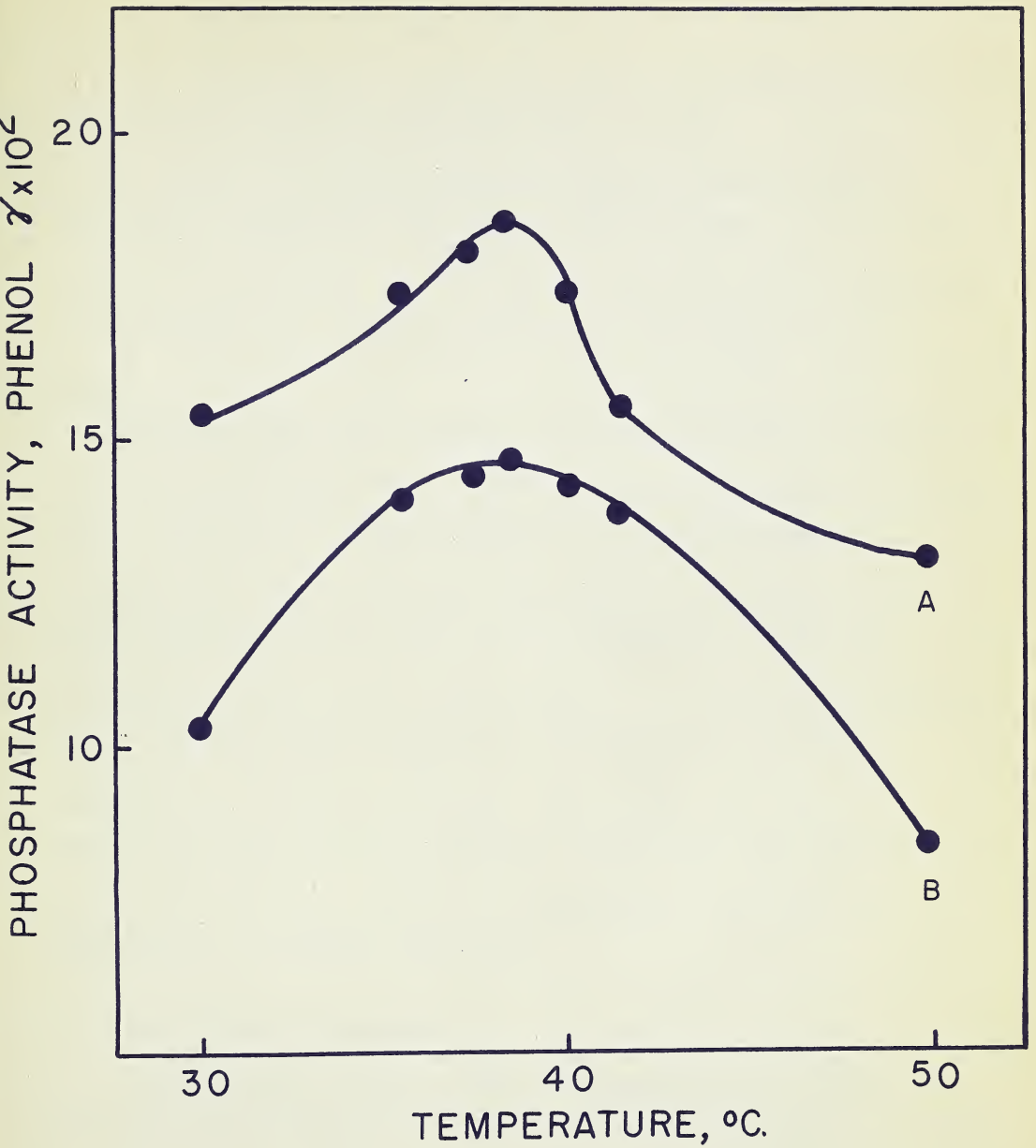


Fig. 8. Apparent temperature optimum for phosphatase activity of pooled milk with two substrate concentrations.

A - 0.029190 M,

B - 0.008340 M.

(c) Optimum Hydrolysis Time

The experimental conditions for the determination of the optimum reaction time were the following: average pH 10.32, substrate concentration 0.02919 M disodium phenyl phosphate, hydrolysis temperature 38.3 $\pm$0.2°C. A series of samples was prepared and the enzyme was allowed to hydrolyze the substrate for varying periods ranging from 5 to 75 minutes.

Table IX shows the results for two milks from individual cows and one pooled milk. Reaction velocities for the three series started to decrease after approximately 25 minutes. The data for the pooled milk are plotted in Fig. 9. The results given in Table IX and Fig. 9 indicate that the hydrolysis period in the determination of phosphatase should not be greater than 20 minutes for the most accurate estimation of enzyme activity. No attempt was made to investigate the several possible reasons for the decrease in activity such as decreased temperature stability or inhibition by reaction products.

(d) Order of Reaction

Experimental evidence has been presented in section II. (a) that the hydrolysis of 0.08340 M disodium phenyl phosphate by alkaline phosphatase of raw milk does not follow a zero order reaction. Because this concentration is recommended by

TABLE IX

OPTIMUM TIME OF HYDROLYSIS OF

DISODIUM PHENYL PHOSPHATE BY PHOSPHATASE

Hydrolysis time, minutes	Phosphatase activity, γ phenol			Average reaction velocity during consecutive 5-minute intervals, γ phenol/second		
	Milk source			Milk source		
	Cow 1	Cow 2	Plant A	Cow 1	Cow 2	Plant A
5	38	60	144	0.127	0.200	0.480
10	77	121	291	0.130	0.203	0.490
15	116	182	435	0.130	0.203	0.480
20	156	243	581	0.133	0.203	0.487
25	196	303	724	0.133	0.200	0.477
30	234	362	861	0.126	0.197	0.457
35	271	421	994	0.123	0.197	0.443
40	307	478	1121	0.120	0.190	0.423
45	342	534	1247	0.116	0.186	0.420
50	376	589	1367	0.113	0.183	0.400
55	409	642	1480	0.110	0.177	0.377
60	441	695	1584	0.106	0.177	0.346
75	-	840	1875	-	0.161	0.323

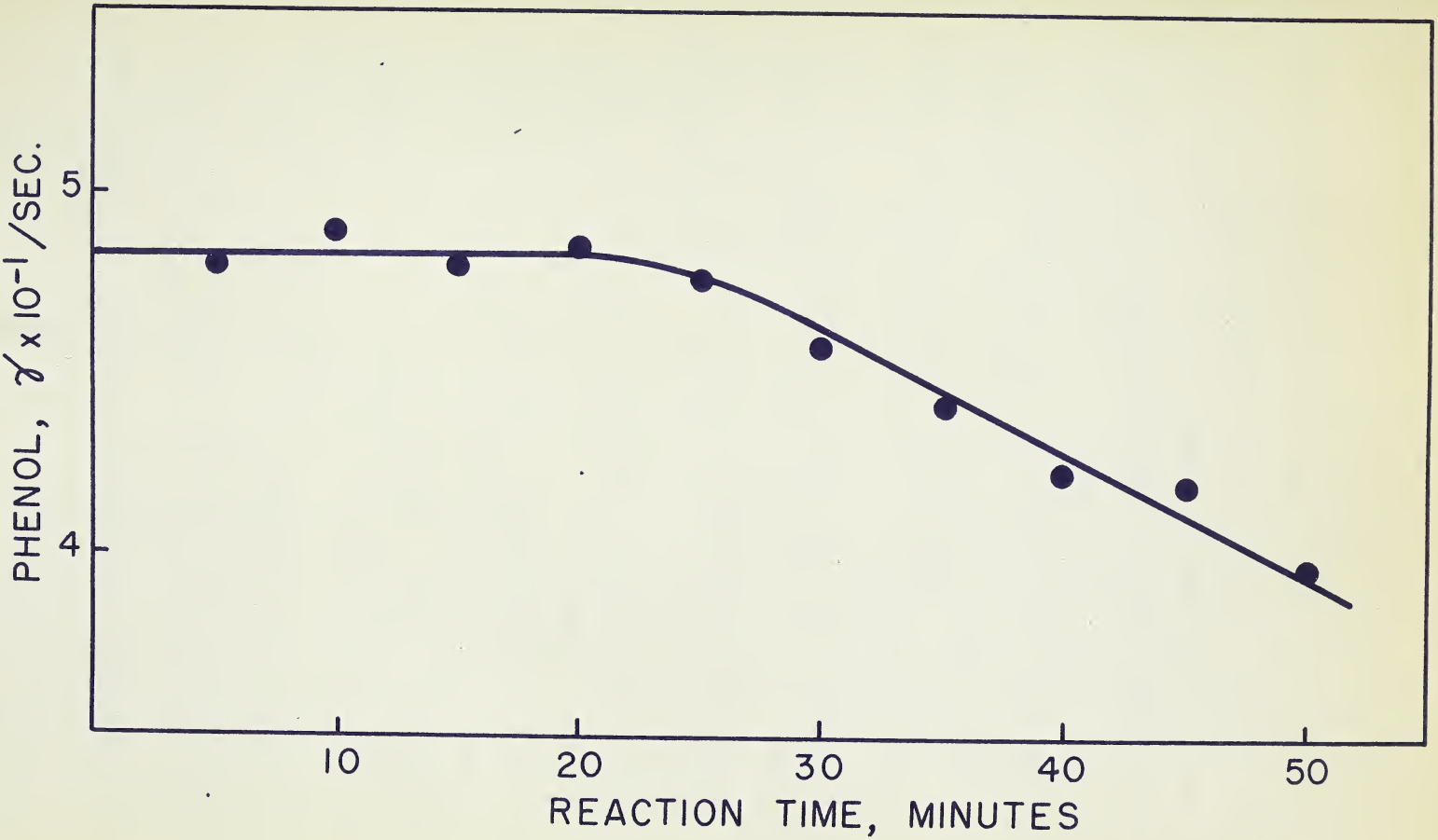


Fig. 9. Rate of hydrolysis during consecutive 5-minute intervals.

Sanders and Sager, it was deemed necessary to compare the reaction orders obtained from different substrate concentrations. Therefore, the rate of hydrolysis and the logarithm of the concentration, respectively, were plotted against time in order to obtain information on the reaction order. Two milks from individual cows and one pooled milk were used in these experiments. The hydrolysis period ranged from 5 to 75 minutes and the reaction pH was 10.0.

Fig. 10 and 11 illustrate the types of curves obtained from the determinations carried out with pooled raw milk. Similar results were found with the milks of individual cows. Fig. 10 shows that a substrate concentration of 0.009174 M disodium phenyl phosphate in buffer or of 0.008340 M in 11 ml. reaction mixture did not provide a zero order reaction. The enzyme apparently was not saturated by this specific concentration of substrate. The decrease in activity during the first 30 minutes of hydrolysis was less pronounced than in the second half of the period. This conforms with the observation made by Hansen who reported less activity in the second half of the period.

Fig. 11 shows that the reaction is not first order, which would present a straight-line relation. It is possible that the reaction order may be intermediate between first- and zero orders for the above experimental conditions. The type

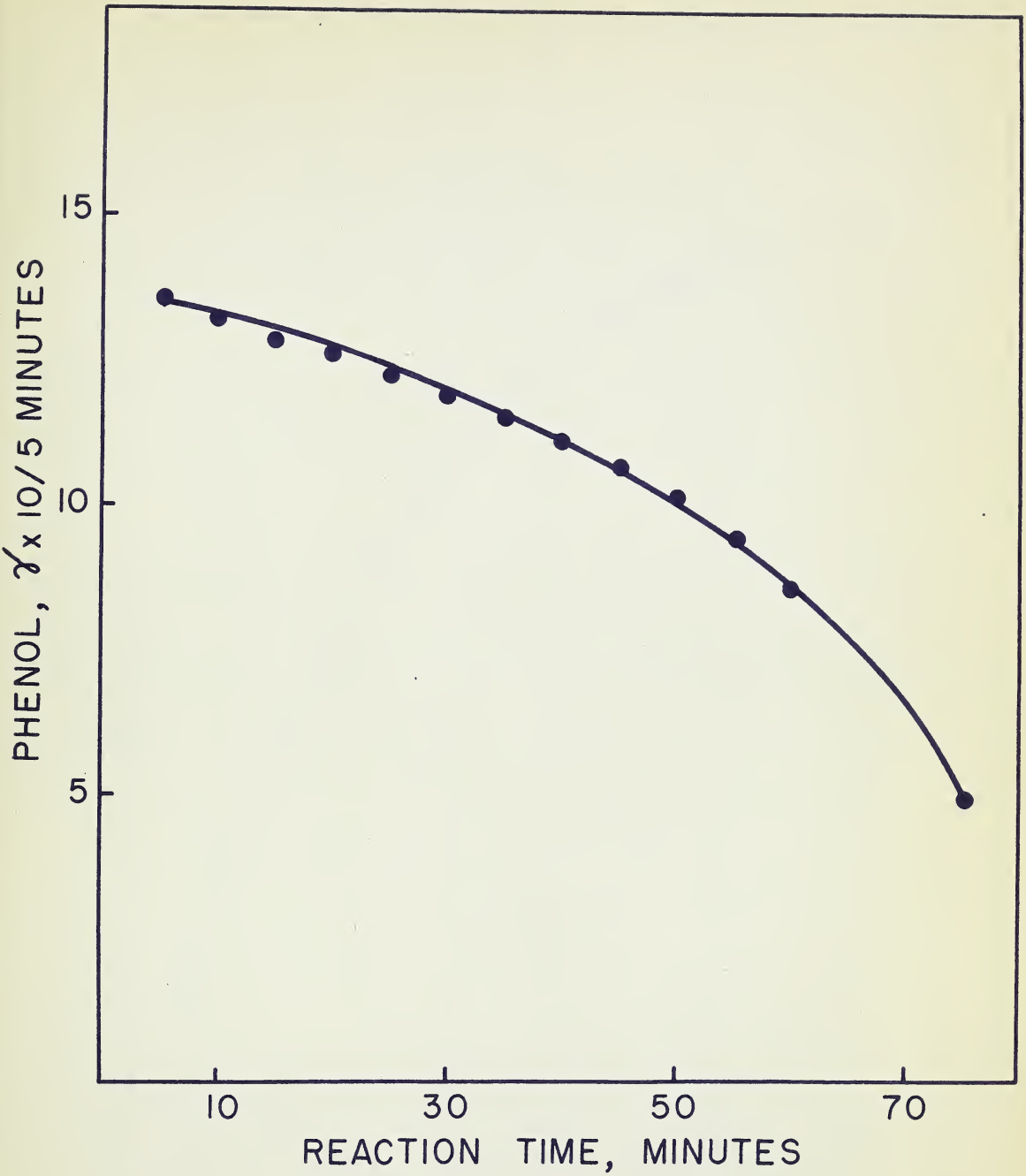


Fig. 10. Phosphatase activity during consecutive 5-minute intervals.

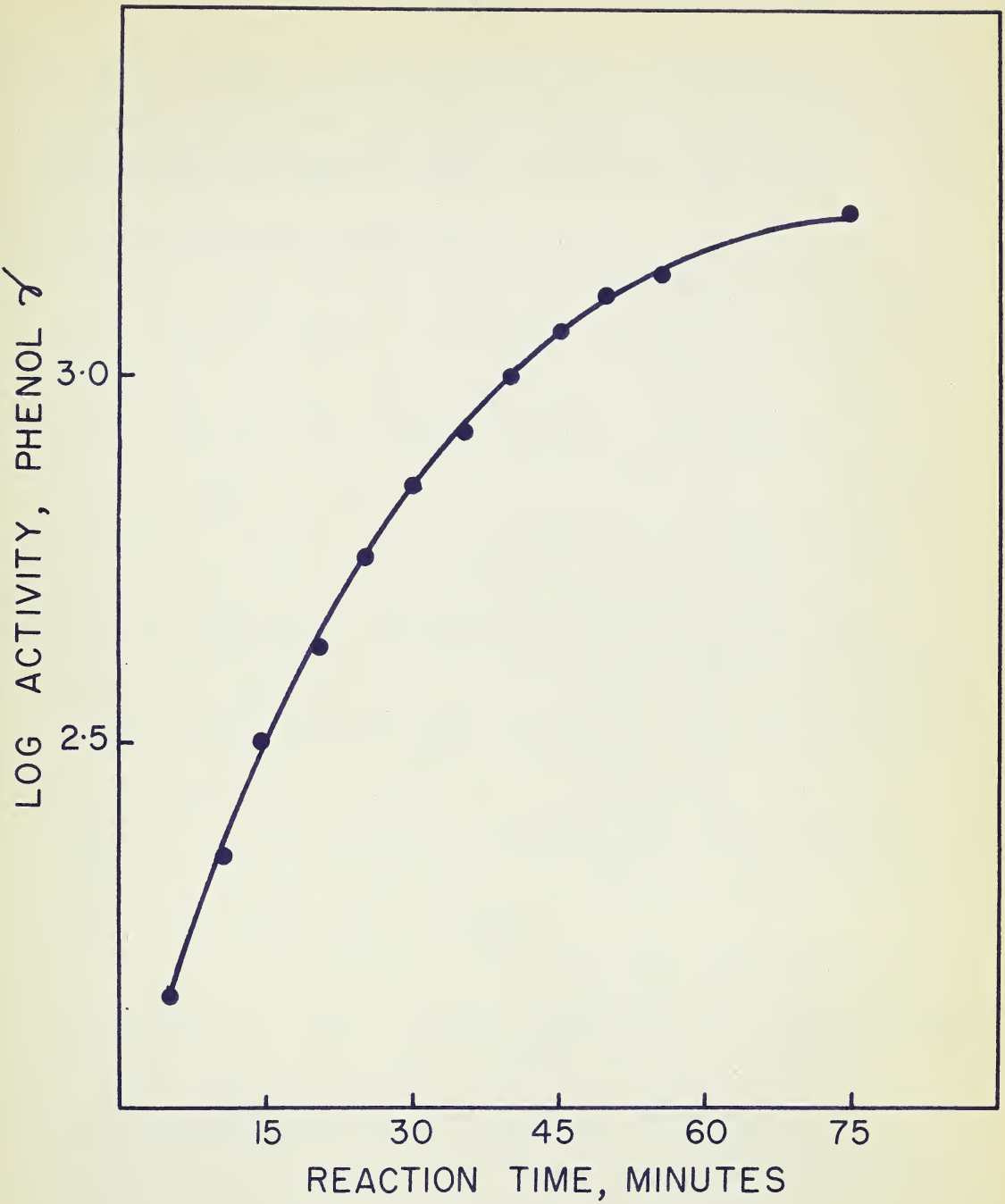


Fig. 11. Plot of logarithm of phosphatase activity against reaction time.

of curve obtained with optimum substrate concentration yielding a zero order reaction, during a limited period of hydrolysis, was shown in Fig. 9 earlier in this thesis.

(e) Michaelis Constants

There seems to be no doubt that the rate of a reaction catalyzed by an enzyme is related to the properties of the intermediate complex between enzyme and substrate. In simple instances of enzymatic catalysis, the reaction velocity might be equal to the rate at which the complex is produced, the rate of complex decomposition or the rate at which products of reaction diffuse away from the enzyme surfaces. Michaelis and Menton (51) postulated that the velocity of the reaction is proportional to the concentration of the complex, which in turn is related to the concentration of the enzyme and its substrate. In many cases it is found that, with a fixed quantity of enzyme, the initial reaction velocity increases with increase in substrate concentration until a limiting value is reached.

For the determination of the Michaelis constant, K_s, it is important to provide optimum conditions for enzyme activity. In cases where an enzymatic reaction is known to follow zero order kinetics, as in the present investigation, conventional methods may be employed to determine Michaelis constants rather than the parameter method of Fleischer (18).

An attempt has been made to express the affinity between alkaline phosphatase of milk and various substrates. Sodium β-naphthyl phosphate could not be used because of its limited solubility in the barium borate-hydroxide buffer. Disodium phenyl phosphate and sodium β-glycerophosphate were employed as substrates. The evaluation of K_s was based on experimental data obtained from reactions occurring at pH optima corresponding to the different substrate concentrations. The experiments with both substrates were carried out using the same raw milk and under optimum conditions.

The sodium β-glycerophosphate substrate required the development of an adequate technique. The procedure used was similar in principle to Gould and Schwachman's modification of the method employed by Shinowara et al. for the determination of alkaline phosphatase activity of blood serum. Preliminary experiments established a convenient sample size, optimum substrate concentration and optimum hydrolysis pH, temperature and time. Addition of different amounts of $MgSO_4$ did not influence the speed of the hydrolysis. The range of different substrate molarities failed to produce the shift of pH optimum observed with disodium phenyl phosphate. The smallest substrate concentration to give optimum conditions for maximum reaction velocity was found to be 0.024601 M sodium

β-glycerophosphate in the reaction mixture. The pH optimum was 9.6 ±0.03 for 11 different substrate concentrations ranging from 0.004139 to 0.038702 M.

In the procedure adopted, 0.5 ml. milk was added to 9.5 ml. buffer substrate, consisting of sodium β-glycerophosphate in barium borate-hydroxide buffer. Each reaction mixture was adjusted to pH 9.6 ±0.03 and held at 37.5°C. for 30 minutes. Then 0.5 ml. reaction mixture was added to 4.5 ml. trichloroacetic acid and each sample centrifuged 15 minutes at 3,000 r.p.m. A suitable amount (0.2 ml. or more) of the water-clear supernatant fluid was made up to 6.0 ml. with 0.05 N NaOH and further diluted if necessary. The color was developed in the usual way by the addition of 2 ml. molybdic acid followed by 2 ml. stannous chloride. The blanks were treated in the same way but were prepared with distilled water instead of milk. Phosphatase activity was expressed as γ phosphorus (as phosphate) liberated by 1 ml. milk under the above conditions.

Graphical determination of Michaelis constants is shown in Fig. 12. The constant was 0.00338 for disodium phenyl phosphate and 0.00560 for sodium β-glycerophosphate. These results indicate that the alkaline phosphatase of milk has a greater affinity for disodium phenyl phosphate than for sodium β-glycerophosphate.

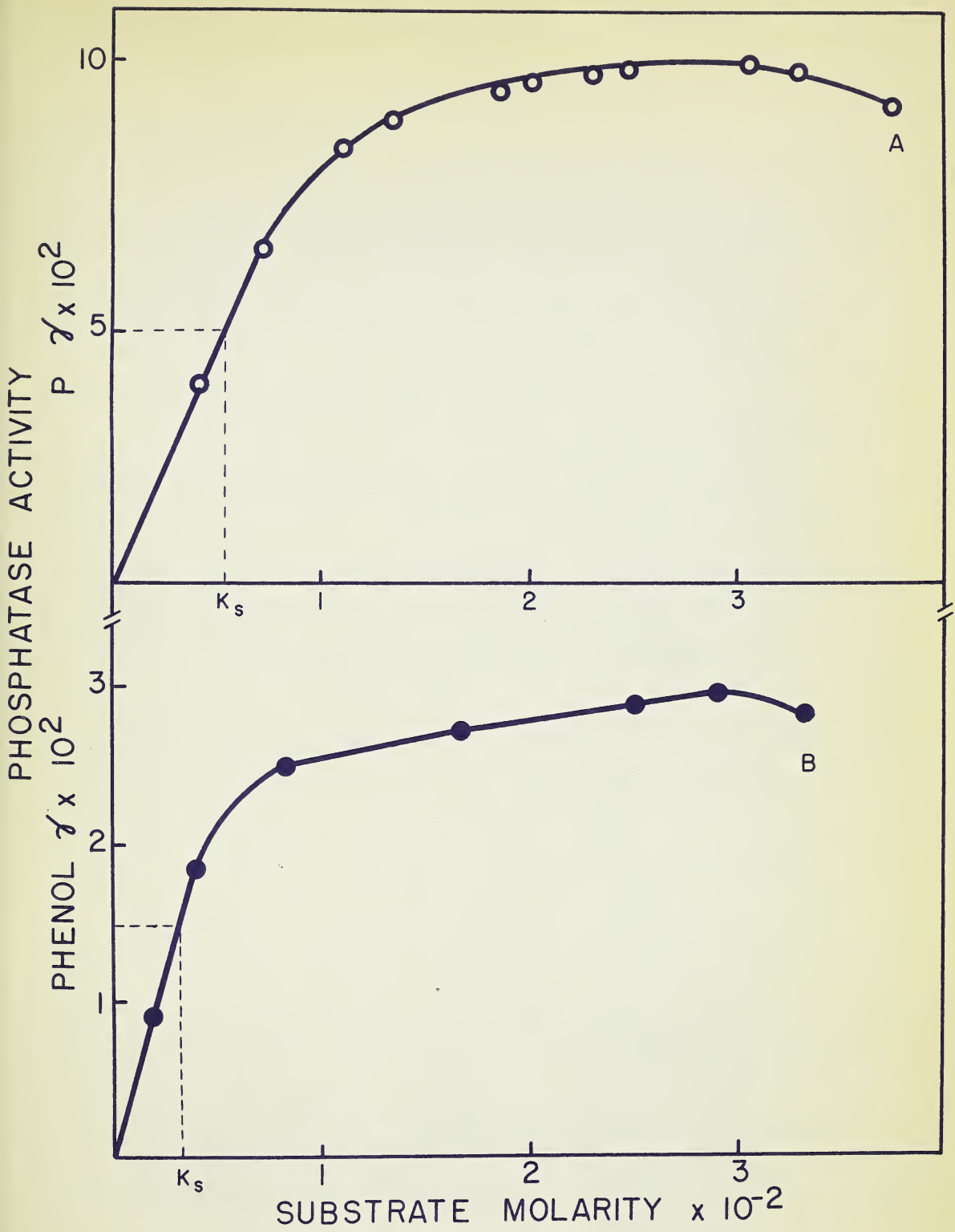


Fig. 12. Graphical determination of Michaelis constants for sodium -glycerophosphate (A) and disodium phenyl phosphate (B).

(f) Energy of Activation

Numerous studies on reaction rates applied to enzyme catalysis have brought forward the concept of activated complex. This refers to an activated state of molecules which have come together and may undergo reaction. The complex has an energy greater than that of the normal reactants. The average of this energy increment is the activation energy.

In 1889, Arrhenius (4) derived an empirical equation to express the thermodynamical nature of chemical reactions including coagulation of proteins. Therefore, the equation has been applied to enzyme reactions. In rearranged form, the equation gives the energy of activation E directly:

$$E = \frac{2.303 \times R \times T_2 \times T_1}{T_1 - T_2} \times \log \frac{K_2}{K_1}$$

where K_1 and K_2 are the rates of reaction at the corresponding Absolute temperatures T_1 and T_2. This formula has been used for the calculation of the activation energy of the hydrolysis of disodium phenyl phosphate by alkaline milk phosphatase.

The substrate concentrations in the reaction mixtures gave first order reactions with each milk sample. The optimum hydrogen ion concentration of the catalyzed reaction was determined preliminarily and found to be approximately pH 9.75 in all cases. The specific reaction velocities at 27° and 37°C. were determined by using hydrolysis periods of 150 seconds and 30 minutes. Another velocity determination at 32°C. served

to check the linear relationship demanded by the Arrhenius equation. The short hydrolysis time necessitated rapid heat destruction of phosphatase and this was accomplished by placing the reaction tubes in a hot sulfuric acid bath.

Table X summarizes the results of experiments on two individual milks and one pooled milk. Fig. 13 shows the plot of log K against 1/T for the milk of cow No. 4. A graphical determination of the activation energy was in good agreement with calculated results obtained with the Arrhenius equation. The observed velocities conformed with the linear relationship demanded by the equation. The ratio of log K to 1/T multiplied by 4.57* yielded an activation energy of 9005 cal. per gram-mole.

The Q_{10} value of 1.4 reported here is within the range of 1.4-2.0 generally quoted for hydrolytic enzymes, but is greater than the 1.2 found by Kannan and Basu (35). The activation energy of 8970 cal. per gram-mole obtained in the present investigation compares more favorably with the finding of Kannan and Basu (35) than with the results of Hansen. He found 10,352 cal. per gram-mole, whereas Kannan and Basu (35) reported energies of activation of 8500, 7600, 8500 and 15,500 cal. per gram-mole for cow, buffalo, goat and sheep milk, respectively. The activation energy of bone phosphatase found

*2.303 x R

TABLE X

ENERGY OF ACTIVATION OF HYDROLYSIS OF DISODIUM PHENYL PHOSPHATE BY MILK PHOSPHATASE

Milk Source	Q_{10}	Log $\frac{K_2}{K_1}$	E cal. per gram-mole
Cow No. 2	1.41	0.20800	8868
Cow No. 4	1.39	0.21276	9071
Plant B	1.40	0.21085	8990
Average	1.40	0.21053	8976

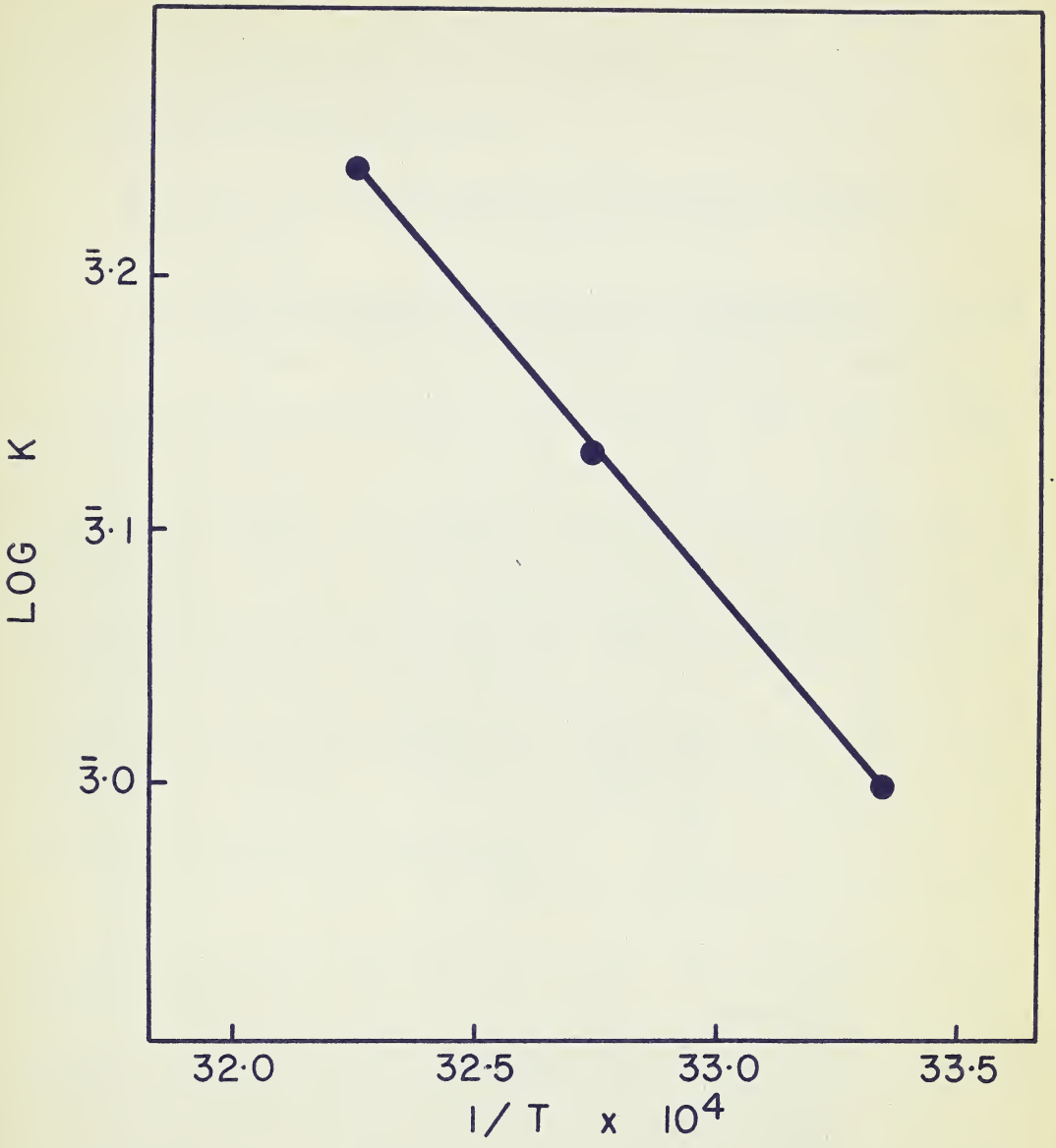


Fig. 13. Plot of the logarithms of the velocity constant K against the reciprocals of the Absolute temperature for the hydrolysis of disodium phenyl phosphate by phosphatase of milk.

by Bodansky (12) was 9870 ±160 cal. per gram-mole for the cat and 10,140 for the human. Madsen and Tuba (48) found an average of 14,500 ±380 for the activation energy of rat intestinal mucosa phosphatase.

(g) Effect of pH on Stability of Enzyme in Absence of Substrate

1) Acid and alkali inactivation of phosphatase. Several investigators have pointed out that the so-called pH-curve is not a true characteristic of an enzyme. The shape of the pH-curve may depend on several factors, such as enzyme preparation, nature of buffer, substrate concentration and temperature. Studies on the effect of pH on the stability of the enzyme in absence of substrate must be separated from studies on the pH of the catalyzed reaction. Milks of two individual cows and a sample of pooled milk were used for a study of the relation between hydrogen ion concentration and the stability of phosphatase.

In order to obtain the different experimental pH values at which the milks were held for 1 hr.- and 2 hr.-periods, 0.1 N NaOH and 0.1 N HCl were added to 10-ml. portions of each milk sample. Enough acid or alkali was added to bring the original pH of 6.6 to 6.8 to approximately 5, 6, 8 and 9.5. Aliquot amounts of each set, corresponding to 1 ml. milk, were placed in four test tubes. Two of these stoppered tubes were held for one hour in a water bath at the apparent temperature optimum

of 38°C., and were gently agitated. At the end of the holding period an appropriate amount of buffer substrate was added to one tube to bring the volume up to 11 ml. The other tube served as a blank and was heated to approximately 85°C., then put back into the water bath and the buffer substrate, previously warmed ~~up~~ to 38°C., was added. Amounts and strength of the barium borate-hydroxide buffer had to be determined in preliminary experiments with the same milk sample in order to obtain the optimum pH of the catalyzed reaction. The substrate concentration used was 0.029190 M disodium phenyl phosphate in the reaction mixture. After 20 minutes of hydrolysis, the tubes were placed in~~to~~ boiling water for approximately one minute, cooled to room temperature, and the color developed in the usual manner. Another series of milk samples with adjusted pH values were held two hours at 38°C. and then treated as above.

Fig. 14 shows that alkaline phosphatase of milk undergoes a fairly rapid inactivation at 38°C. on either side of the zone of its natural pH. When the samples were kept at the stated pH values for only one hour instead of two hours, a flatter peak was obtained. Martland and Robinson (49), King (42) and Folley and Kay (19) made somewhat similar observations for bone, kidney and mammary gland tissue phosphatases, respectively. Therefore, the general effect of pH on the stability of the enzyme in the absence of substrate is similar for phosphatases

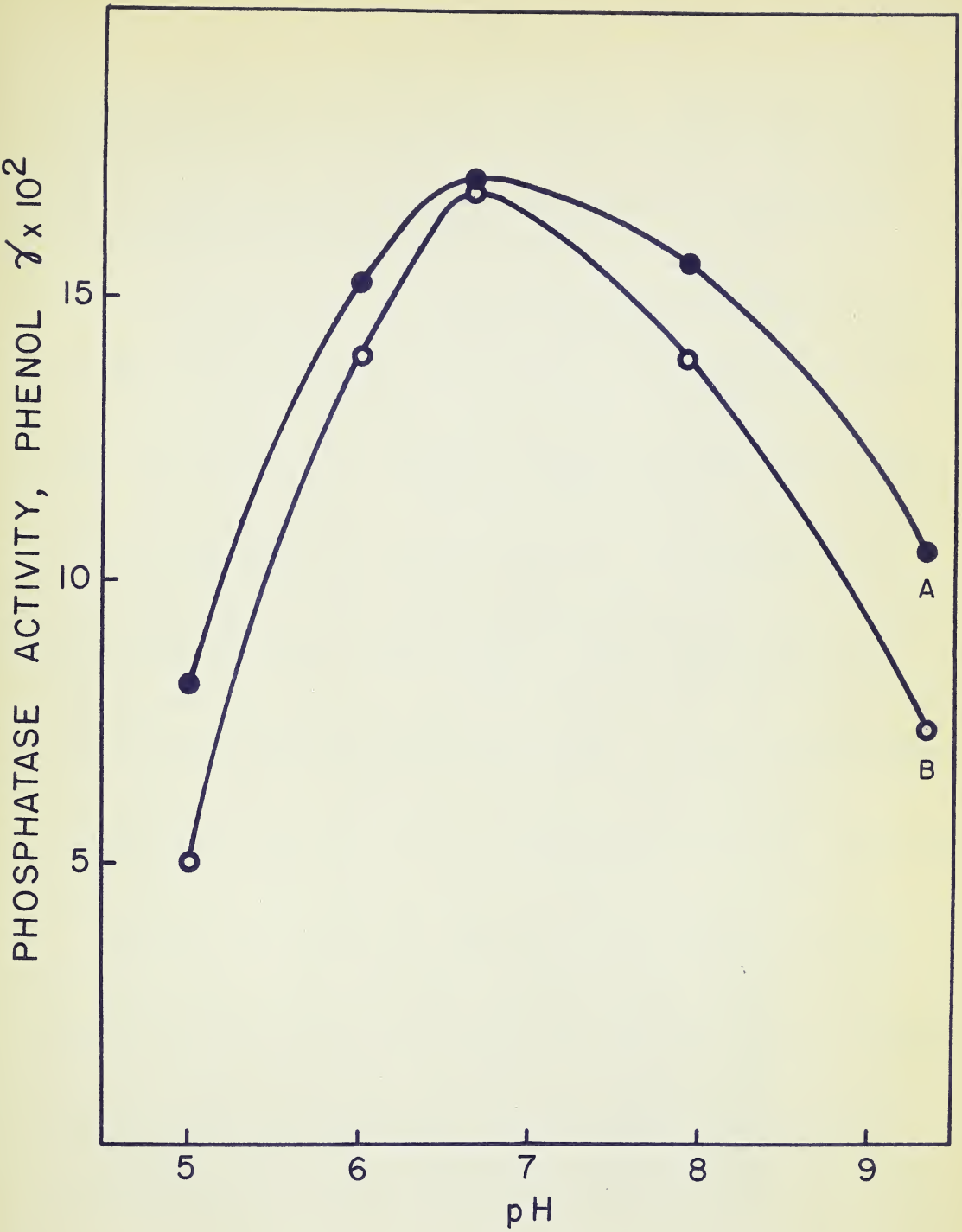


Fig. 14. Partial inactivation of phosphatase held at different pH values for 1 hr. (A) and 2 hr. (B).

of milk and these other sources. However, there seem to be slight differences in whether more rapid inactivation takes place at the acid or at the alkaline side of the pH of maximum stability, which is approximately pH 7.

2) Restoration of phosphatase activity. It might be well to make brief mention of the so-called "reactivation" of heat- or acid-inactivated alkaline phosphatase of cow's milk. In 1935, Herschdorfer (30) made the observation that heat-treated milk regained phosphatase activity after being cooled slowly to room temperature in about 45 minutes. Heat-treated milk cooled quickly to 5°C. showed a lower phosphatase content than the same milk cooled slowly in air. These results are the reverse of what would be expected, namely, that the samples slowly cooled and thus maintained longer at higher temperatures, would have lower phosphatase content. This investigator presumed that heating does not destroy the phosphatase completely but causes an inactivation which is partially reversible.

Wright and Tramer (75) studied the phosphatase activity of commercial samples of milk of Swiss origin, sterilized by rapid heating to high temperatures, and observed the development of a positive phosphatase reaction on storage. The investigators called this phenomenon "reactivation". In a further investigation (76) they showed that there was a clear relation

between reactivation and the denaturation of the soluble proteins of milk as indicated by Aschaffenburg's turbidity test. In addition, they found the presence of reducing conditions was favorable to reactivation. Reheating of heat-shocked milks created conditions more favorable to reactivation.

Sjostrom studied the pH optimum for reactivation of phosphatase activity of acid-treated cow's milk. The reactivation optimum was located at about the natural pH of the enzyme and not at pH 9, which was the pH optimum of the catalyzed reaction of the K-G-S phosphatase method. In addition, the investigator showed that a rapid souring or direct addition of acid lowered the obtainable reactivation as compared with slow souring.

In the present investigation, phosphatase activity of a pooled milk was determined. A portion of this milk was kept at room temperature and reached pH 5.2 after 14 hours. Another portion of the same milk was kept at 5°C. and brought to pH 5.2 with 0.1 N HCl after 14 hours. Production of bacterial phosphatases was considered unlikely (72). Both samples were preserved with 1.5% chloroform and held at 37°C. for two hours. Then the residual phosphatase activities were determined and portions of both samples brought to pH 6.7 and 9.5 with 0.1 N NaOH. After six hours at 37°C., the

phosphatase activities were again determined. Blanks for each step were prepared and treated according to the requirements of the procedure.

The results, summarized in Table XI, are in agreement with the findings of Sjostrom. The milk samples kept at pH 6.7 for six hours regained more of their lost phosphatase activity than those held at pH 9.5.

A study of the literature on reactivation phenomena suggests that it is not possible to find a final answer to the problems at the present state of knowledge. However, several tentative explanations have been advanced. Albers (1) studied the acid inactivation of yeast and kidney phosphatases. He observed an almost complete reactivation of phosphatase activity after bringing the preparations back to optimum pH for a certain length of time. Albers assumed that dissociation and association of apoenzyme and coenzyme were caused by lowering and increasing the pH, respectively. Sjostrom based his disagreement with Albers' theory upon the observation that the pH optimum for the reactivation of milk phosphatase lies at the natural pH of the enzyme and not at the considerably higher pH optimum of the catalyzed reaction. If Albers' theory were applied to the results obtained on either side of the natural pH of the enzyme, it could provide an alternative explanation for any of the observations made by Sjostrom,

TABLE XI

RESTORATION OF ACTIVITY OF ACID-INACTIVATED PHOSPHATASE OF POOLED MILK*

Acidity increased	Time to reach pH 5.2	Percent residual activity after 2 hr. at pH 5.2	Percent restoration after 6 hr. at	
			pH 6.7	pH 9.5
By souring at 20°C.	14 hr.	32.7	19	7
By 0.1 N HCl	15 sec.	28.6	9	3.5

*Original activity: 1650 γ phenol.

who explained the phenomenon on the basis of restoration of the original hydration of the enzyme protein at its natural pH. Hansson et al. (29) found that the pH optimum for reactivation of enzyme concentrates is shifted towards pH 8 in apparent accordance with Albers' theory. A mixture of phosphatase concentrate and pasteurized milk, soured and reactivated, showed a shift back to a reactivation optimum at pH 6.5. On the other hand, raw skim milk, whey, and cream had their optimum reactivation at pH values higher than the pH 6.5 for whole milk. In this connection it should be remembered that the heat resistance of phosphatase of moisture-free milk preparations is abnormally high. It is evident that the degree of hydration affects the properties of milk phosphatase in several ways. The Swedish workers mentioned above suggest a relation between the pH of optimum reactivation of phosphatase activity and the purity of the enzyme. In addition, they see the possibility of various fractions of the milk proteins acting as apoenzymes for the cophosphatase.

Wright and Tramer (76), made numerous investigations and advanced the three following tentative explanations for the reactivation of heat-inactivated milk phosphatase:
1) Reactivation occurs as a result of the reversion of the denaturation of the apoenzyme taking place under the influence

of reducing conditions produced at temperatures above 70°C. 2) Heat inactivation of milk phosphatase is a result of the destruction of the original coenzyme. A new coenzyme formed by the heat treatment takes its place being closely connected with the production of reducing systems. 3) Heat inactivation of milk destroys an essential link between apoenzyme and coenzyme. In reactivation, the apoenzyme is again linked to the coenzyme.

All three hypotheses are based on the rupture of the bond between apoenzyme and coenzyme and differences in these concepts are principally in the manner in which this rupture occurs. Moreover, in all three explanations, the association of apoenzyme and coenzyme by means of a new link is assumed to be a time-temperature reaction. A comparison of these ideas with suggestions advanced by others does not reveal extreme differences. Albers' theory is based on a time-pH reaction rather than a time-temperature relation. Sjostrom's conception of a restoration of original hydration after an acid treatment does not necessarily exclude any other possible explanation. His hypothesis of various fractions of milk proteins serving as apoenzymes is, in principle, not different from the coenzyme replacement suggested by Wright and Tramer.

Sjostrom did not put forward an explanation for the hindering effect of rapid souring upon reactivation of acid-inactivated milk phosphatase. It might be well to point out here that Herschdorfer observed a similar effect caused by rapid cooling of heat-treated milk. In both cases an increased reactivation resulted where a decrease was expected. The important criterion seems to be the suddenness of change rather than actual length of exposure to the inactivating factors, e.g., hydrogen ion concentration or heat. The process of breaking the essential link between apoenzyme and coenzyme may have to overcome a moment of inertia. The more abruptly this happens the greater will be the effect.

Final and satisfactory solutions for the various problems involved in inactivation and reactivation of alkaline milk phosphatase will require further research.

Furthermore, it is evident that the heat-inactivation of phosphatase at pasteurization temperatures is a problem which is difficult to solve by merely considering the time-temperature relations because different and changing factors, depending on the milk and the enzyme itself, are affecting the process.

(h) Effect of Miscellaneous Compounds on Phosphatase Activity

The increased or decreased activity of an enzyme, caused by a reagent present in relatively small amounts, is a well-known phenomenon. Action of enzyme inhibitors has been

studied by numerous investigators. Singer (64) divided enzyme inhibition into the three following categories: (a) reduction of the effective concentration of the prosthetic group, (b) competition with the substrate for the enzyme, and (c) destruction of an essential functional group in the protein moiety of the enzyme. According to this investigator, there is no doubt about the importance of the application of enzyme inhibitors, as group-specific protein reagents, to studies on the action mechanisms of enzymes. Sizer (66) divided the mechanism of enzyme inhibition into the five following groups:

1. By reaction with the active groups of the protein part of the enzyme.
2. By reaction with other groupings of the enzymes.
3. By combination with the coenzyme or activating cation.
4. By reaction with the enzyme-substrate complex.
5. In the living organism, by preventing the cell from synthesizing the specific enzyme protein (apoenzyme) or the necessary coenzyme.

Although the interference with enzyme synthesis caused by an inhibitor is doubtless of primary importance in the explanation of certain types of biological activity of inhibitors, it does not apply to their action on isolated enzyme systems. The general properties of such a system may be altered by an inhibitor.

Various investigators report several substances to have either an inhibitory or an activating effect on phosphatases. Grier et al. (25) and Klemperer et al. (44) found beryllium to inhibit certain phosphatases. Massart and Vandendrsiessche have shown that the alkaline phosphatase of milk is a metal-protein complex in which a heavy metal like zinc is included rather than magnesium or manganese. Kelly (41) reported an increased activity of milk phosphatase after an addition of zinc in low concentration. Kannan and Basu (34) found that zinc in 10^{-6} M concentration is neither an inhibitor nor an activator and concluded that the milk might have contained zinc in sufficient concentration for optimum enzyme activity. It has been observed that the manganese and zinc ion concentrations in milk are low and will vary widely with different samples. Graham and Kay (24) and Kay (37) report the activation of milk phosphatase by magnesium ion. The latter, working with serum phosphatase of rabbits, found stimulating effect on the hydrolysis of sodium β-glycerophosphate by magnesium ion in low concentration and an inhibitory effect in higher concentration. Kay (37) also studied the inhibitory effects of acid addition and dialysis, and the reverse effects obtained by additions of alkali or the dialysate. Cloetens (15) found that inhibitory effects can be caused by very small concentrations of cupric ions with the formation of slightly ionized complexes.

The inhibitory effect of penicillin on the alkaline phosphatase of milk was illustrated by Stoltz (69) who claimed that amounts of penicillin in raw milk up to a level of 1 unit per ml. can be detected by their effect on the phosphatase. A special method based on phosphatase inhibition for the determination of penicillin in milk has been reported by Gogas (22).

Zittle and Della Monica (77) studied the effect of sodium tetra borate on milk phosphatase and showed that this enzyme was much less inhibited than the mucosa enzyme of the cow. Both enzymes were inhibited competitively by borate, probably by an ionic effect. Milk phosphatase was found to be inhibited non-competitively by ethanolamine.

Belfanti et al. (8) who found increased activity of milk phosphatase at pH 10 in the presence of either magnesium or manganese ions, hold the view that metal-enzyme complex formation is a preliminary to activation. They state further that the magnesium cannot be displaced from the metal enzyme complex in the form of phosphates as suggested by Kannan and Basu (34). These investigators reported an inhibitory effect of zinc in concentrations higher than 10^{-6} M. Not one of 15 amino acids accelerated phosphatase activity. Experimental evidence, found in the literature, for activation of bovine milk phosphatase refers only to cases where enzyme concentrates and dialyzed or diluted milk samples were used in the tests.

1) Activation. Activation of alkaline phosphatase of raw milk by magnesium ion may be an important factor in providing optimal experimental conditions. Because the milk samples were not diluted or dialyzed and no enzyme concentrates were used in this investigation, it was considered unlikely that addition of magnesium ion would result in increased hydrolysis. Nevertheless, the phosphatase activity of milk was determined with various amounts of magnesium ion added to the reaction mixtures.

The substrates used were disodium phenyl phosphate in barium borate-hydroxide buffer and sodium β-glycerophosphate in both barium borate-hydroxide and veronal buffers. The tests where borate buffer was used were carried out essentially the same way as described in section (e). However, the pH values were checked in order to provide the specific optimum hydrogen ion concentrations. Preliminary determination of optimal experimental conditions, using veronal buffer, led to the adoption of the following technique.

A 0.5 ml. milk sample was added to 8.5 ml. buffer substrate (0.024601 M sodium β-glycerophosphate in the final reaction mixture) and 1 ml. water or magnesium chloride solution. Different molarities of veronal buffer provided the reaction pH optimum of 9.8. Hydrolysis at 38°C. was stopped after 30 minutes by making up 0.5 ml. reaction mixture

to 10 ml. with 20% trichloroacetic acid. The tubes were centrifuged for 10 minutes at 2500 r.p.m., then 0.5 ml. of the water-clear supernatant was added to 5.5 ml. 0.1 N NaOH. Blanks were prepared the same way except that the reaction was stopped at zero time.

Table XII shows the results obtained from a series of experiments on an individual milk. Similar results were obtained with pooled milk. The original activities of individual and pooled milks were approximately 1500 γ phenol. The presence of 0.001 M $MgCl_2$ or $MgSO_4$ in the reaction mixture did not affect hydrolysis. However, 0.01 M $MgCl_2$ or $MgSO_4$ caused a drop in activity up to 20% of original values. These data, as well as preliminary results, indicate that magnesium ion was not a limiting factor in either individual or pooled milks.

2) Inhibition. An attempt was made to determine the effects of sodium oxalate, sodium taurocholate and sodium cyanide upon the hydrolysis of disodium phenyl phosphate and sodium β-glycerophosphate by phosphatase. Barium borate-hydroxide buffer was used in the determination of phosphatase by the hydrolysis of disodium phenyl phosphate as described in section (e). When sodium β-glycerophosphate was the substrate, veronal was employed as buffer under the experimental conditions described above. The barium borate-hydroxide buffer excluded the use of sodium oxalate and sodium cyanide in the

TABLE XII

EFFECT OF MAGNESIUM ION UPON THE PHOSPHATASE ACTIVITY OF INDIVIDUAL MILK

Buffer	Substrate	Molarity		Percent of original phosphatase activity
		$MgCl_2$	$MgSO_4$	
Barium borate-hydroxide	Sodium β-glycero-phosphate	0.001	--	99.6
		0.01	--	84.6
"	Sodium phenyl phosphate	--	0.001	101.8
		--	0.01	81.3
Veronal	Sodium β-glycero-phosphate	0.001	--	99.7
		0.01	--	79.4

experiments with disodium phenyl phosphate because of interfering reactions.

Table XIII summarizes the average results of four or more experiments with each molarity of inhibitor on the same individual or pooled milks. The phosphatase activity level of each milk was approximately 1500 γ phenol. The data show that 0.001 M oxalate did not have a significant inhibitory effect. However, the same concentration of cyanide caused a sharp drop in activity and taurocholate also partially inhibited the enzyme. Further experiments similar to the above, except that 0.001 M $MgCl_2$ was present in the reaction mixtures, showed that the degrees of inhibition were not affected by the change of magnesium ion concentration.

The degrees of inhibition obtained in the present investigation are in fair agreement with the data of Madsen and Tuba (47) for bone and kidney phosphatases of normal rats.

TABLE XIII

EFFECT OF INHIBITORS ON PHOSPHATASE ACTIVITY OF MILK WITH TWO DIFFERENT SUBSTRATES

Inhibitor	Molarity in Reaction Mixture	Residual phosphatase activity, percent of original values	
		Disodium phenyl phosphate	Sodium β-glycerophosphate
Oxalate	0.01	--	83.7 ±1.7
	0.001	--	98.1 ±2.0
Taurocholate	0.001	81.8 ±2.0	80.3 ±1.9
Cyanide	0.01	--	15.4 ±1.6
	0.001	--	37.2 ±2.1

GENERAL DISCUSSION

In the present investigation the experimental results have been discussed in each section of the thesis but further consideration of certain aspects may be desirable.

Ritter (57) and Hansen found that the method of Sanders and Sager did not provide optimal conditions for the estimation of phosphatase in raw milk. Results obtained in the present investigation confirm the observation of Ritter and Hansen. The accuracy of the phosphatase assay was increased by shortening the hydrolysis period to 30 minutes and improving the estimation of phenol.

The calibration curve for the Sanders and Sager methods for both raw and pasteurized milk are not prepared under the conditions used in conducting the test. Therefore, possible variations attributed to the effect of heat and the presence of milk, buffer substrate and protein precipitant are not eliminated by the blank. Moreover, in the present investigation, known amounts of phenol added to milk were only partly recovered; but the data did indicate a straight-line relation. It has not been established that a constant portion of the phenol released by the enzyme is always found in the determination.

The variation in phosphatase activity of pooled milk might influence the degree of heat destruction of the

enzyme during pasteurization, because heat denaturation of proteins can be affected by concentration of reactants. The destruction of phosphatase by heat has received considerable attention by other investigators, but studies on the time-temperature relation of phosphatase inactivation are not in full agreement. Procedures for the determination of residual phosphatase must provide for standardization of all factors which affect the activity of the enzyme, except the phosphatase concentration. Optimal conditions for enzyme activity, fundamental to evaluation of reported data, have been determined in the present study.

Certain other factors may be important in the study of phosphatase remaining in milk subjected to heat. It is not clear whether the residual enzyme is identical with the original phosphatase or is a more heat-resistant fraction. The results of Morton suggest that variations in the nature of lipoproteins associated with phosphatase could explain differences in heat stability of phosphatase molecules. The phenomenon of phosphatase reactivation in milk subjected to high temperatures has been observed by Tramer and Wright. They reported that reducing conditions favored the reactivation. Although Tramer and Wright did not observe reactivation in pasteurized milk, it is possible that pasteurization and subsequent storage could create conditions favorable for reactivation of the enzyme.

Folley and Kay (20) concluded that milk phosphatase originates from the mammary gland and that it has probably no relationship with the blood serum phosphatase. Wilson and Hart suggested some elimination of serum phosphatase through the milk because low activity was found in the plasma of heavily producing cows. In the present investigation the fast drop of phosphatase activity of colostrum to a minimum during the time when milk secretion is becoming normal could be explained by elimination of blood phosphatase and the presence of blood or parts of blood in the colostrum. However, the possibility that the enzyme is formed in the tissue of the udder would indicate that the presence of the enzyme in the milk is not due to simple elimination of serum phosphatase through the milk. Thus, it is possible that milk phosphatase is connected with the phosphorus metabolism involved in milk secretion. The findings of Chanda and Owen (14), who analyzed the phosphorus and thiamine partition in cow's milk throughout a normal lactation, lend support to this view. Data obtained by Crookshank et al. (16) show that the level of alkaline serum phosphatase of the cow remains relatively constant for an individual animal. This fact also seems to indicate local biosynthesis of milk phosphatase in the tissue of the mammary gland.

Hansen found a Michaelis constant of 0.003 for phosphatase of milk, which is slightly less than the value of 0.00338 found

in the present investigation. It can be further observed that with decreasing pK of the substrate there is an increase in hydrolytic activity. Thus the affinity between enzyme and substrates increases with decreasing pK of the substrate. These observations can be interpreted in terms of protection given to the enzyme against hydroxyl ions, as suggested by Delory and King (17). On this basis, the observed shift of pH optimum with changing substrate concentration could be explained.

Albert (2) made quantitative studies of the avidity of naturally occurring substances for trace metals and worked with amino acids with two or three ionizing groups. On the basis of his work, the activation of alkaline milk phosphatase by zinc ion might indicate the presence of amino acids having high affinity for zinc ion, especially of cystein. Barron and Singer (7) stated that, among other metallo-proteins, acid phosphatase was found to require no SH-groups for enzyme activity. Kannan and Basu (34) found considerable activation of enzyme concentrates by magnesium ion in the case of cow's milk. However, magnesium ion did not activate goat, sheep or buffalo milk phosphatases. On the basis of Klotz's (45) explanation of enzyme-substrate complex formation, the striking differences between the degrees of interaction of magnesium or manganese ion and alkaline phosphatase of sheep,

goat, buffalo and bovine milk might also indicate differences in properties of the enzyme protein. His concept of metal-stabilized activated state in enzymatic hydrolysis would explain the joint requirement of metal and specific protein, the juxtaposition of metal and substrate being a relatively uncommon occurrence in the absence of protein. It is not clear for these cases whether an actual prosthetic group is required for the formation of the activated enzyme-substrate complex. Moreover, evidence presented by Kutscher and Sieg (46) suggested that the prosthetic group of alkaline phosphatase probably is choline pyrophosphate.

The results of the present investigation are consistent with the view of Jaquet and Saignt (33) that different bovine milks contain the same alkaline phosphatase. Kinetic data obtained are in fair agreement with values for bone and kidney phosphatases (47, 77, 78).

CONCLUSIONS

1. The phosphatase test for raw milk suggested by Sanders and Sager does not provide optimal experimental conditions.

2. The modified Sanders and Sager method used for routine determinations in this study gave a more accurate estimation of phosphatase activity.

3. Limited data indicated that only part of the phenol added to raw and pasteurized milks could be recovered in the phosphatase determinations. This fact could be of importance in pasteurization control.

4. Phosphatase activity of milks from four individual cows ranged from 70 to 4,400 γ phenol per 0.5 ml. milk during approximately one lactation period.

5. The initial high activity of colostrum decreased very rapidly but after approximately 10 days the activity of the milk gradually increased. During the middle part of the lactation period, milk yield decreased whereas phosphatase activity increased. The change from summer to winter feeding did not result in unusual variations in phosphatase activity.

6. Data were obtained which were consistent with the view that phosphatase of milk originates in the mammary gland and is not derived directly from serum phosphatase.

7. Phosphatase activity of pooled milks from a large number of cows ranged from 950 to 1700 γ phenol during the period May 27, 1953 to March 15, 1954. The activity curve followed the trend observed for individual cows. Fluctuations in phosphatase activity were not related to variations in fat content.

8. Differences in the amounts of phosphatase remaining in milk after pasteurization cannot be attributed solely to differences in the time-temperature treatment.

9. The optimum pH of the catalyzed reaction was dependent on the substrate concentration, among other factors.

10. At 37.5°C. the optimum hydrolysis of disodium phenyl phosphate was defined by 0.029190 M substrate at pH 10.32 for a hydrolysis period of 20 minutes.

11. The molarity of substrate used in the Sanders and Sager method did not result in either a zero-, or a first, order reaction.

12. The Michaelis constants were 0.00338 for disodium phenyl phosphate and 0.00560 for sodium β-glycerophosphate. These results indicate that milk phosphatase has a greater affinity for disodium phenyl phosphate than for sodium β-glycerophosphate.

13. The Q_{10} value obtained was 1.4 and the average energy of activation was 8970 cal. per gram-mole.

14. In the absence of substrate, milk phosphatase was partially inactivated at 38°C. on either side of the zone of its natural pH. The activity of phosphatase partially inactivated by acid was regained to a limited extent.

15. The presence of 0.001 M $MgCl_2$ or $MgSO_4$ in the reaction mixture did not activate phosphatase, but 0.01 M concentrations decreased phosphatase activity.

16. The inhibitory effect of sodium oxalate, sodium taurocholate and sodium cyanide on milk phosphatase varied with the specific compound and its concentration. Sodium cyanide resulted in the greatest inhibition.

APPENDIX

TABLE A

NAME AND BREED OF COWS FROM WHICH MILKS WERE TAKEN FOR THE PRESENT INVESTIGATION

Cow No.	Name	Breed
1	Annabella	Jersey
2	Queen	Holstein
3	Senora	"
4	Joane	Jersey
5	Alice	Holstein
6	Anna	Jersey
7	Netherland	Holstein
8	Samantha	"
9	Sonata	"
10	Tess	Jersey

TABLE B

ONE EXAMPLE OF VARIOUS BUFFER SETS

USED FOR pH ADJUSTMENTS

$Ba(OH)\cdot 8H_2O$ in 500 ml.* H_2O gm.	H_3BO_3 in 500 ml.* H_2O gm.	Approximate** pH of	
		Buffer substrate	Reaction Mixture
22.5	11	10.14	9.62
23.0	11	10.20	9.76
24.0	11	10.31	9.87
25.0	11	10.55	10.02
23.5	11	10.33	10.01
24.5	11	10.47	10.07
25.5	11	10.60	10.27
27.5	11	10.80	10.33
26.0	11	10.70	10.38
26.5	11	10.77	10.48
25.0	10	10.82	10.52
27.5	11	10.92	10.60
28.0	11	11.04	10.71
28.5	11	11.10	10.78

* Mixed together and further diluted when desired

** Depending on dilution of buffer (and on milk for the reaction mixture)

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